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DAMD 17-82-C-2235

TRICOTHECENES MYCOTOXIN STUDIES

Annual Summary Report

G.H. Buchi, M.A. Marletta, P.M. Newberne,
A.E. Rogers, W.R. Roush, G.N. Wogan

February 1986

Period Covered:
October 1, 1984 - July 31, 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-82-C-2235

Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Introduction

The recurring public health problem associated with tricothecene contaminated cereal grains known for decades, has now been expanded to include the use of these toxins in chemical warfare. The sporadic episodes in the past have been managed largely by diagnosing the disease and removing the source of exposure; little effort has gone into studies designed to determine the basic nature of the biological response. As a consequence we are poorly prepared to identify exposed populations and to intervene with therapeutic or protective measures.

Concerns about real or potential harm this class of toxins can cause in military and civilian populations have resulted in an acceleration of research efforts into the chemistry and biology of the tricothecenes. It is to such concerns that this contract has supported an integrated effort of biologists, chemists and pathologists. During the past period which this report covers (October 1, 1984-July 31, 1985) we have made a number of significant advances in our understanding of the chemistry and biology of anguidine (DAS). Salient features and observations of the various segments are presented here.

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER: DAMD 17-82-C-2235	2. GOVT ACCESSION NO. AD-A168 093 / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)*: Tricothecenes Mycotoxin Studies	5. TYPE OF REPORT & PERIOD COVERED: Annual Summary 10/1/84-7/31/85
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): G.H. Buchi, M.A. Marletta, P.M. Newberne, A.E. Rogers, W.R. Roush and G.N. Wogan	8. CONTRACT OR GRANT NUMBER(s): DAMD 17-82-C-2235
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Massachusetts Institute of Technology 77 Massachusetts Avenue Cambridge, MA 02139	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS: US Army Medical Research & Development Command Fort Detrick, MD 21701	12. REPORT DATE: February 1986
	13. NUMBER OF PAGES: 97
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*	15. SECURITY CLASS. *(of this report)*: U
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

Same as #16.

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Tricothecenes, mycotoxins, protectants

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The purposes of the research supported under this contract are to determine the toxicity of two members of the tricothecene mycotoxins, [anguidine, (DAS) and nivalenol] including their gross and microscopic effects; absorption, distribution and excretion by several routes of administration; to identify and synthesize the more prominent metabolites, and to identify or synthesize compounds which will block or reduce the toxicity of DAS and nivalenol.

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20. During the past year we have developed a method for topical exposure to the mycotoxins in rodents which confirms our observations by other routes of administration that there is a significant difference in sensitivity between rats and mice. The mouse excretes 80% of a dose of DAS within 24 hours, 90% of which appears in the urine. This is in contrast to most literature reports on other tricothecenes (T-2) where the feces is the major route of excretion. A detailed analysis of distribution and excretion by topical application is in progress.

DAS is not a substrate for microsomal cytochrome P450 isozymes, in vitro, but it is a good substrate for microsomal carboxylesterases with major metabolites being 4-acetoxyscirpenediol, 15-monoacetoxyscirpenol and scirpentriol. A major metabolite identified is an anguidine glucuronide conjugated at the C3 position. Most major metabolites appear to be the same in rats and in mice but rates of absorption and excretion vary between the two species.

Other lines of investigation have resolved difficulties in the synthesis and stability of ^{3}H-anguidine, ^{14}C-anguidine and in the synthesis of nivalenol. Two compounds, among other 25 synthesized have exhibited protective effects against DAS and others are under test. We are now testing a number of natural substances including retinoids, antioxidants and selected amino acids.

We have developed a system (colony forming unit) to study stem cells of the bone marrow of rodents exposed to DAS. Long-term effects of DAS on hematology and on immunocompetence are in progress using this system and acute stem-cell effects are under study. An ornithine decarboxylase inhibitor (DFMO) has a protective effect on bone marrow cells but does not appear to affect mortality of rodents exposed to DAS. The CFU-S system has shown that stem cells recover rapidly.

In the rodent the testes is the first and most sensitive organ as an indicator of DAS toxicity and is being used to detect earliest effects and correlate these with metabolites in animals exposed by different routes of exposure including mice, rats and guinea pigs, the latter by the respiratory route which is still in development.

TABLE OF CONTENTS

Introduction----------2

Segment 1A- In Vitro Metabolism----------4

Segment 1B- In Vivo Metabolism----------23

Segment 2A- Chemistry----------33

Segment 2B- Chemistry----------35

Segment 3- Toxicology----------57

References----------93

Segment 1A - *In Vitro Metabolism*

M.A. Marletta

Segment 1A: Metabolism

M.A. Marletta, Principal Investigator

Our goal had been to determine the route(s) of anguidine metabolism in vitro, and to carry out large-scale production of metabolites for structure elucidation. Anguidine has several sites available for metabolism by the isozymes of cytochrome P450. We looked for these metabolites in phenobarbital induced, B-naphthoflavone induced and uninduced rat liver microsomes. Anguidine could potentially be metabolized by epoxide hydrolase, or conjugated with glutathione and glucuronic acid in the presence of glutathione transferase or glucuronyl transferase, respectively. We also investigated these possibilities. Preliminary identification of in vivo metabolites could be made by comparison of chromatographic retention times with the in vitro metabolites.

Anguidine (35-347 μM) was incubated with 1.40, 1.34 and 1.25 μM cytochrome P450, respectively, from phenobarbital, B-naphthoflavone, or uninduced rat liver microsomes in 0.1 M K_2PO_4, pH 7.5 for 1 hour at 37° C, with shaking. An NADPH regeneration system was used. ^{3}H-anguidine (12 μM), synthesized by Dr. Roush's group, was used as a tracer. HPLC was performed with a MeOH/water gradient on a μBondapak C18 reverse-phase column. Fractions were monitored by scintillation counting and pooled for further analysis.

Anguidine is rapidly metabolized. The chromatographic profiles of incubations with the various induced microsomes were identical, except that the product ratios were different (Figure 1 and 2), suggesting that the cytochrome P450 isozymes showed no product specificity. Identical incubations in the absence of NADPH, necessary for cytochrome P450 activity, produced identical chromatograms to those previously observed with NADPH. This suggested that anguidine was not a substrate for cytochrome P450. For further confirmation, we performed inhibition studies by monitoring the effect of anguidine on the O-deethylation of 7-ethoxyresorufin in β-naphthoflavone induced microsomes. No inhibition of benzphetamine N-demethylation was observed, however, anguidine caused inhibition of the O-deethylation of the 7-ethoxyresorufin. This inhibition was found to be non-competitive (Figure 3), therefore, anguidine is not a substrate for the microsomal cytochrome P450 isozymes.

The metabolites were derivatized with bis(trimethylsilyl)trifluoroacetamide for 1 hour at 100° C, concentrated, and analyzed by GC/MS (Figure 4). All peaks except A, B and C were in the control incubation. (The extent of derivatization was monitored by TLC on silica plates, which were developed in EtOAc and visualized by spraying the plates with 5% H_2SO_4/EtOH then heating on a hot plate). The samples were analyzed with a methylsilicone column and both electron impact and chemical ionization modes. Comparison of the sample spectra with that from standards of 4-acetoxyscirpenediol, 15-monoacetoxyscirpenol, and scirpenetriol (prepared by Dr. Roush's group), showed conclusively that these are the major metabolites produced by microsomal incubation of anguidine (Figures 5-7). Anguidine is a good substrate for the microsomal carboxylesterases responsible for this metabolism.

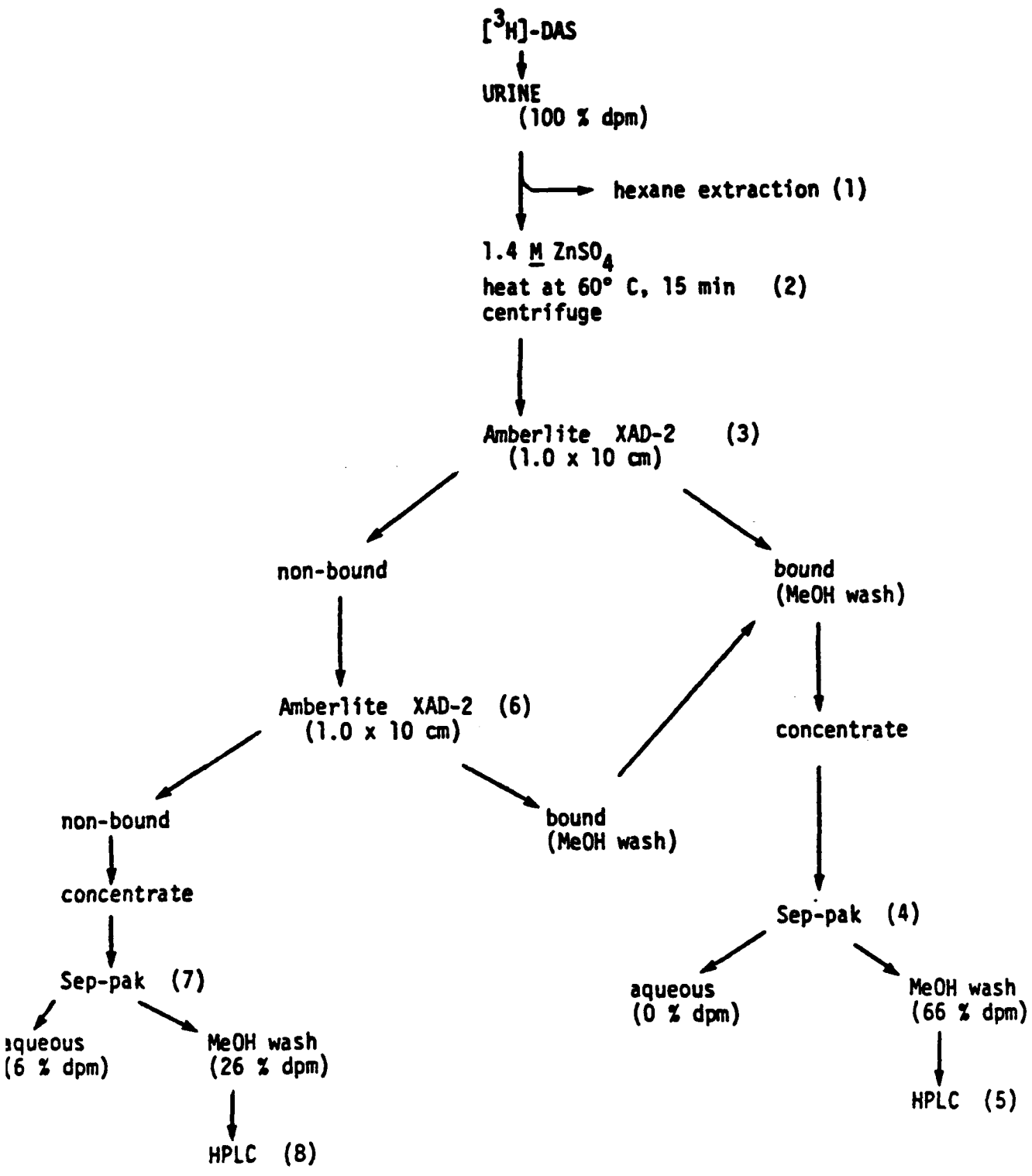

[3H]-DAS
URINE
(100 % dpm)
hexane extraction (1)
1.4 M ZnSO4
heat at 60° C, 15 min (2)
centrifuge
Amberlite XAD-2 (3)
(1.0 x 10 cm)
non-bound
bound
(MeOH wash)
Amberlite XAD-2 (6)
(1.0 x 10 cm)
concentrate
non-bound
bound
(MeOH wash)
concentrate
Sep-pak (7)
Sep-pak (4)
aqueous
(6 % dpm)
MeOH wash
(26 % dpm)
aqueous
(0 % dpm)
MeOH wash
(66 % dpm)
HPLC (8)
HPLC (5)

FIGURE 1

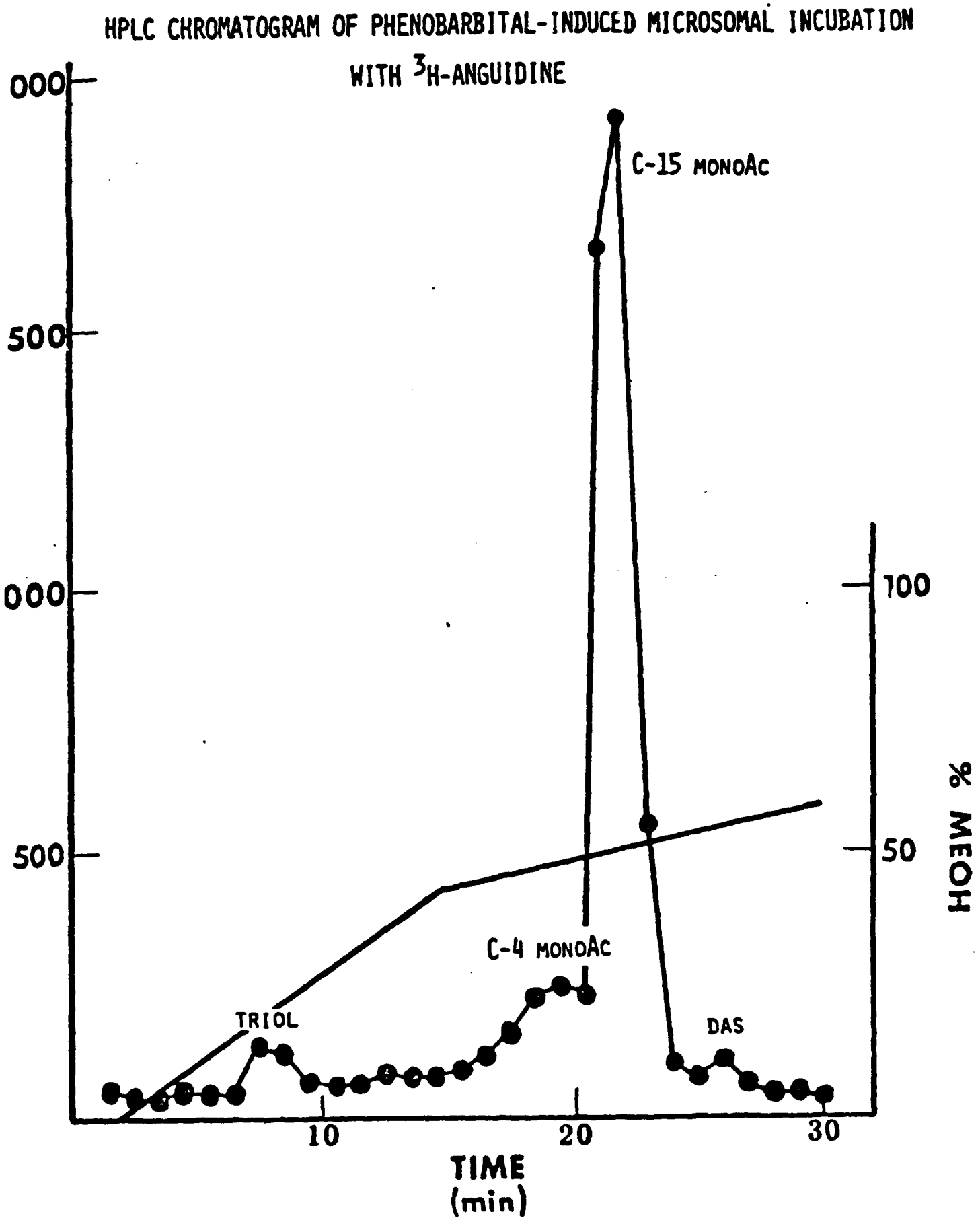

HPLC CHROMATOGRAM OF PHENOBARBITAL-INDUCED MICROSOMAL INCUBATION WITH ^{3}H-ANGUIDINE
000
500
000
500
C-15 MONOAC
C-4 MONOAC
TRIOL
DAS
100
50
% MEOH
10
20
30
TIME
(min)

FIGURE 2

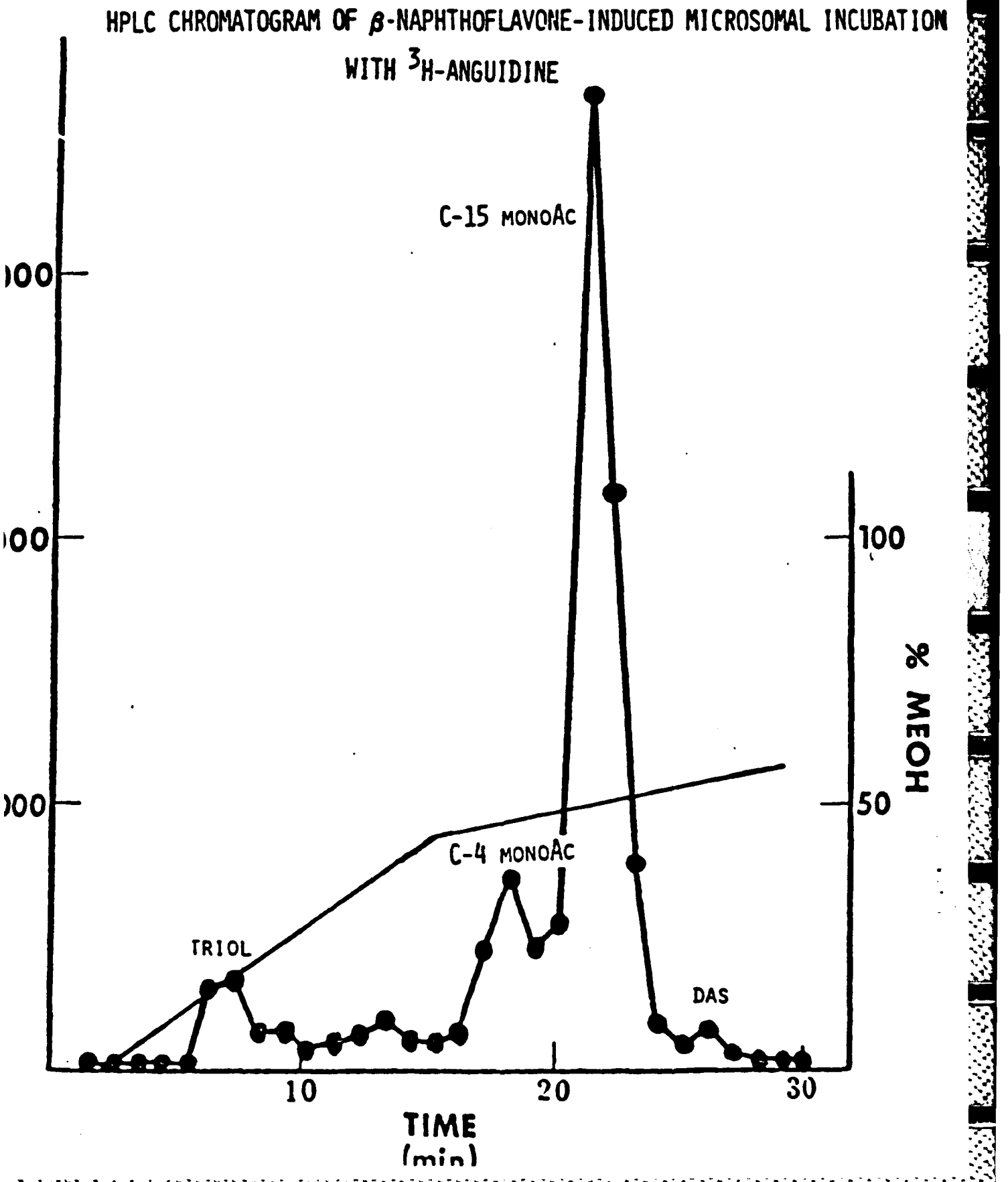

HPLC CHROMATOGRAM OF β-NAPHTHOFLAVONE-INDUCED MICROSOMAL INCUBATION WITH ^{3}H-ANGUIDINE
C-15 MONOAC
C-4 MONOAC
TRIOL
DAS
100
50
% MEOH
10
20
30
TIME
(min)

FIGURE 3

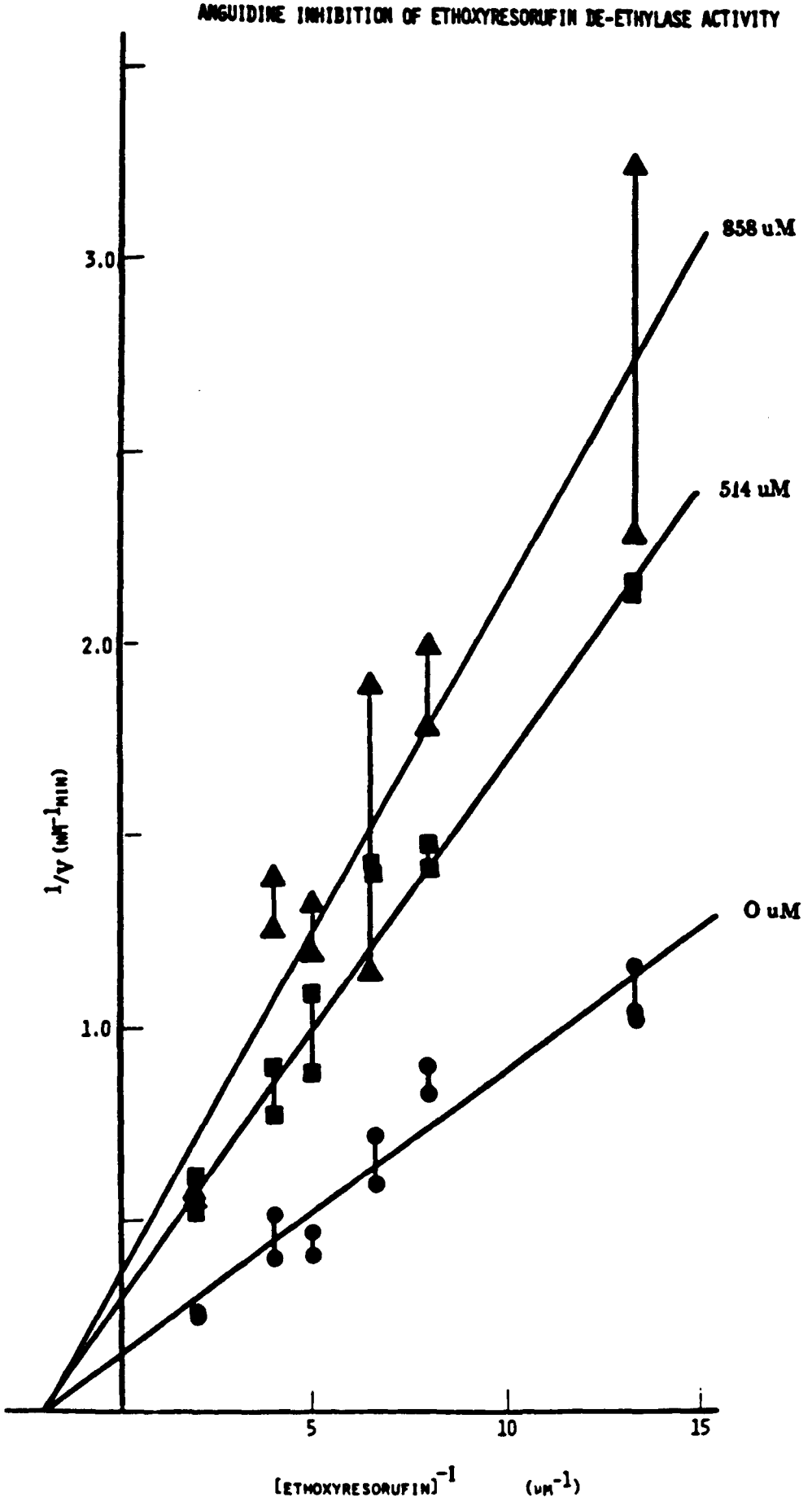

ANGUIDINE INHIBITION OF ETHOXYRESORUFIN DE-ETHYLASE ACTIVITY
858 uM
514 uM
0 uM
3.0
2.0
1.0
1/V (nMT-1MIN)
5
10
15
[ETHOXYRESORUFIN]-1 (uM-1)

FIGURE 4

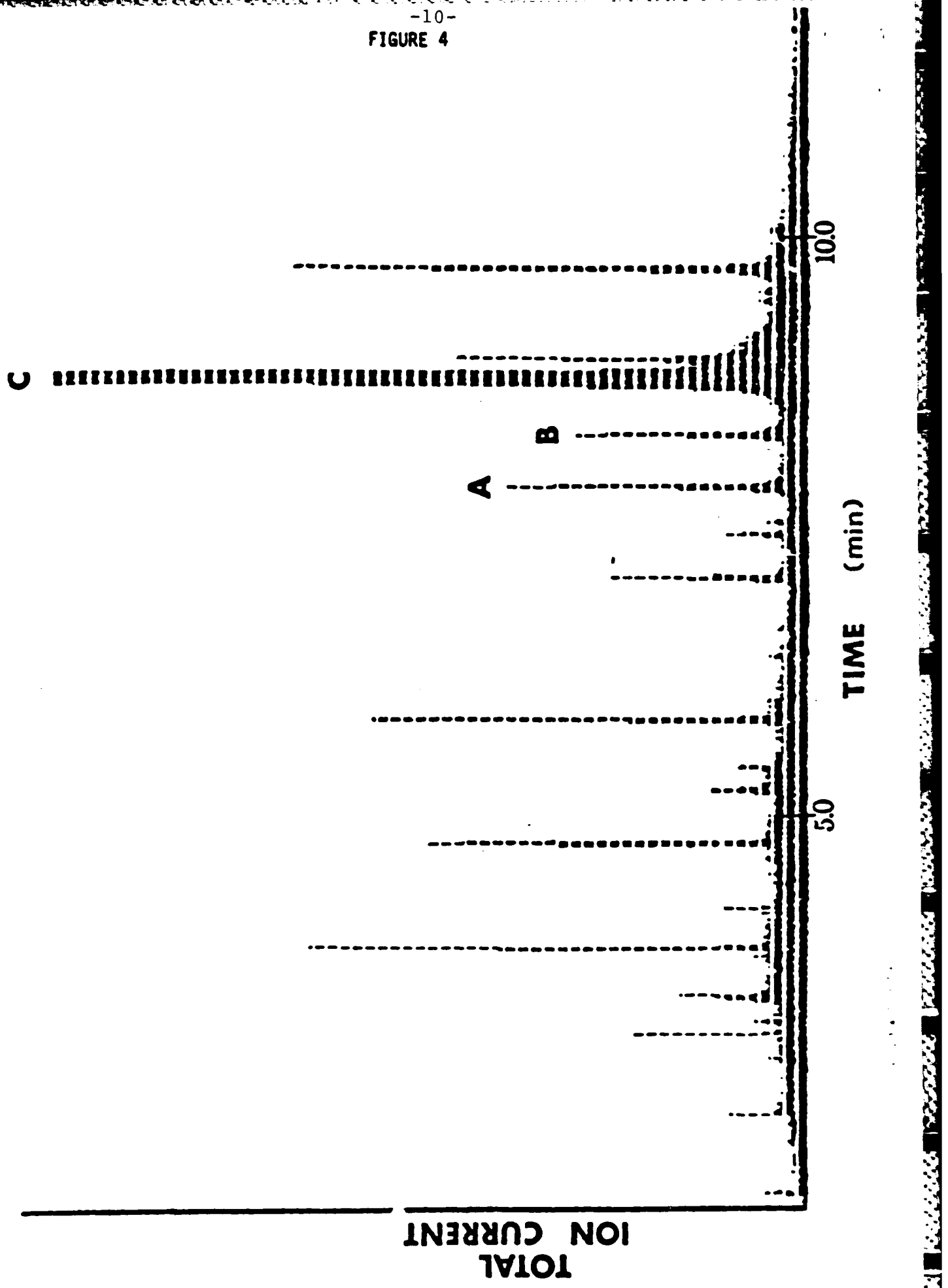
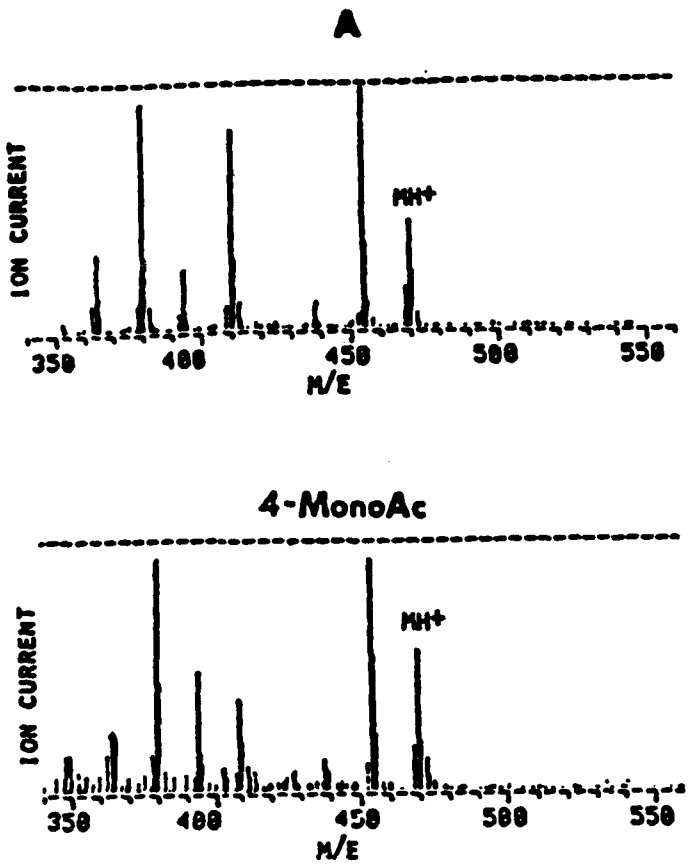
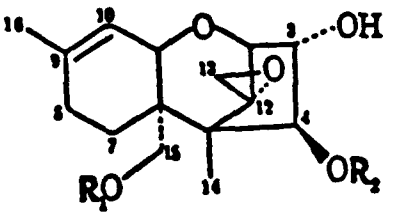


FIGURE 5

CHEMICAL IONIZATION





ELECTRON IMPACT

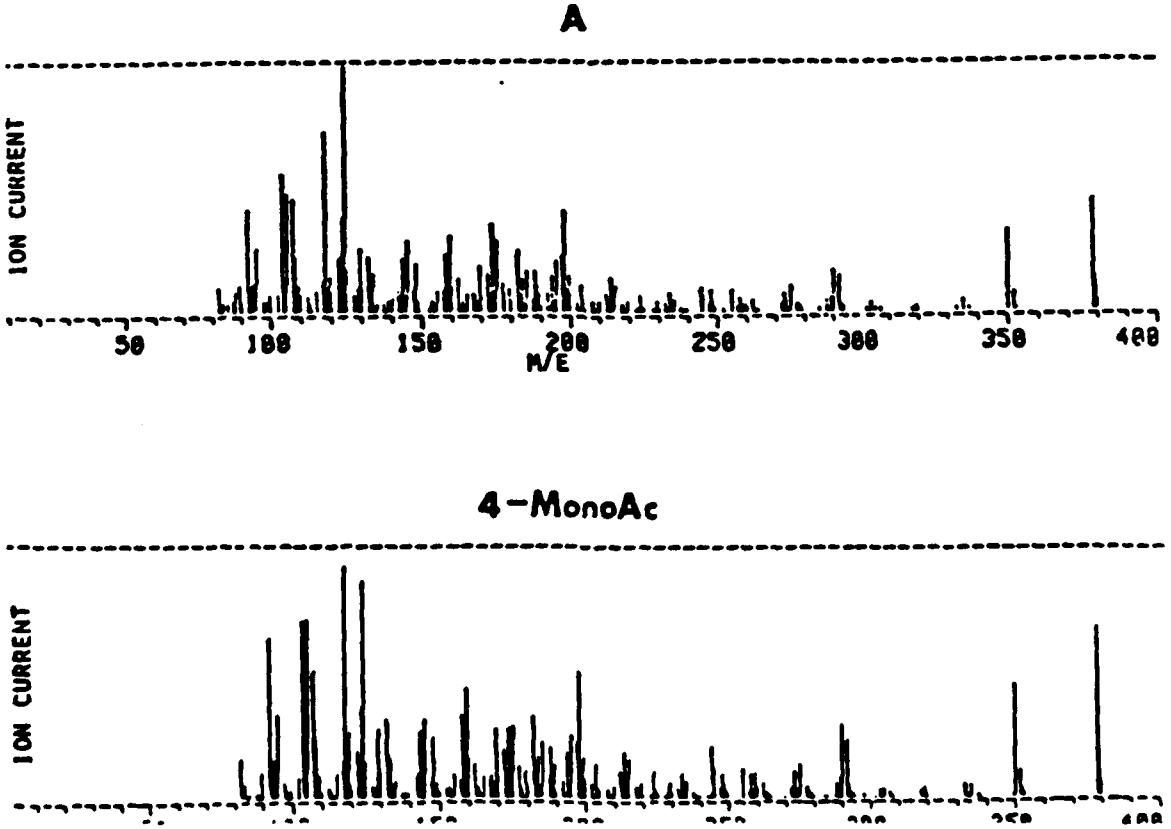
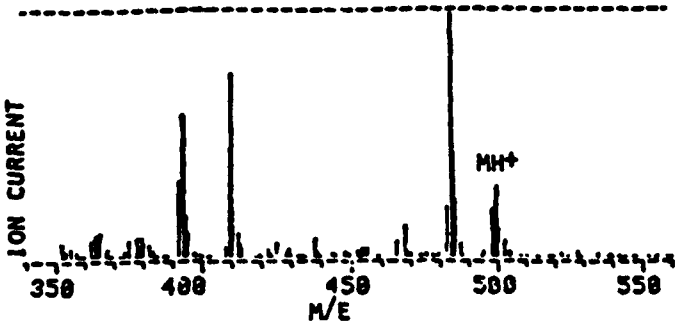


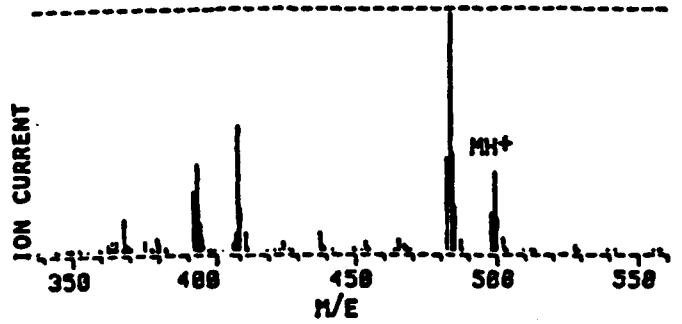
FIGURE 6

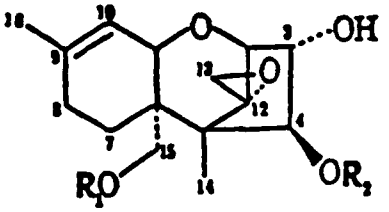
CHEMICAL IONIZATION

B



TRIOL

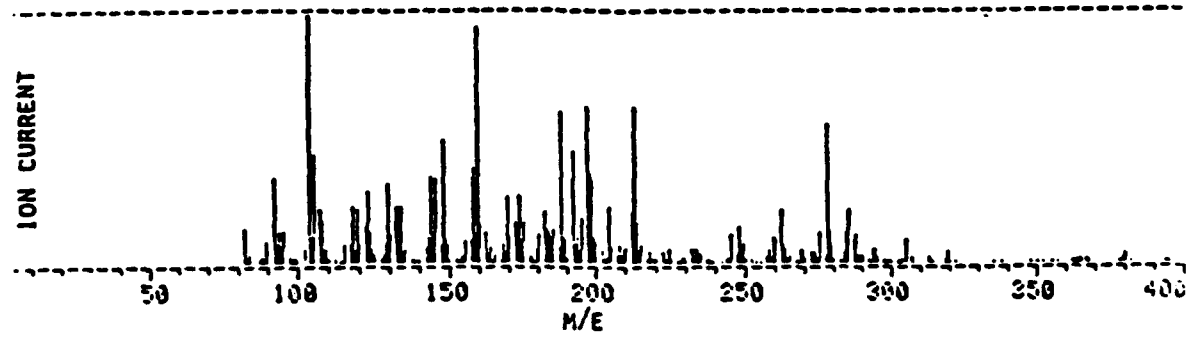




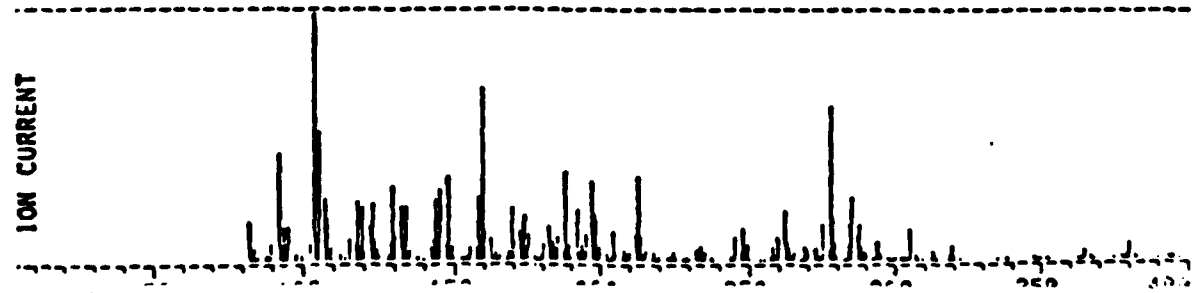
$R_1 = R_2 = H$

ELECTRON IMPACT

B



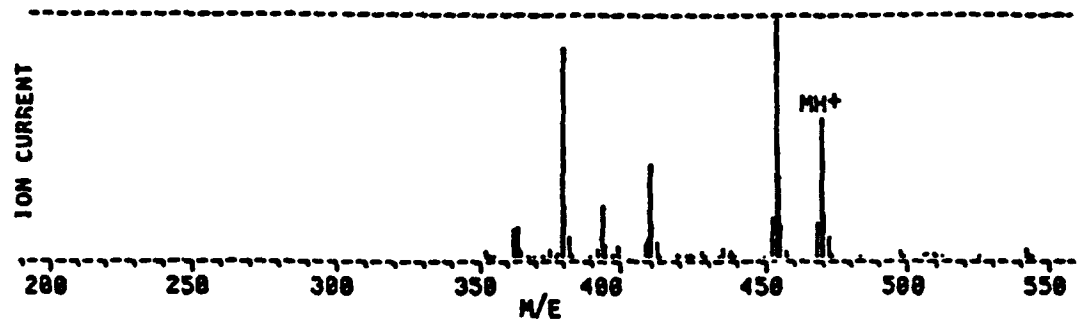
TRIOL



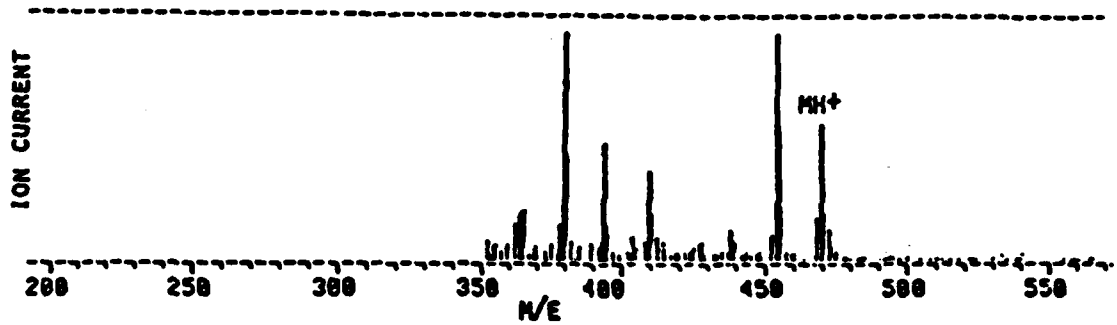
CHEMICAL IONIZATION

FIGURE 7

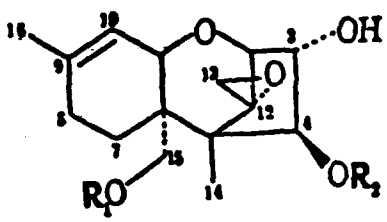
C



15-MonoAc

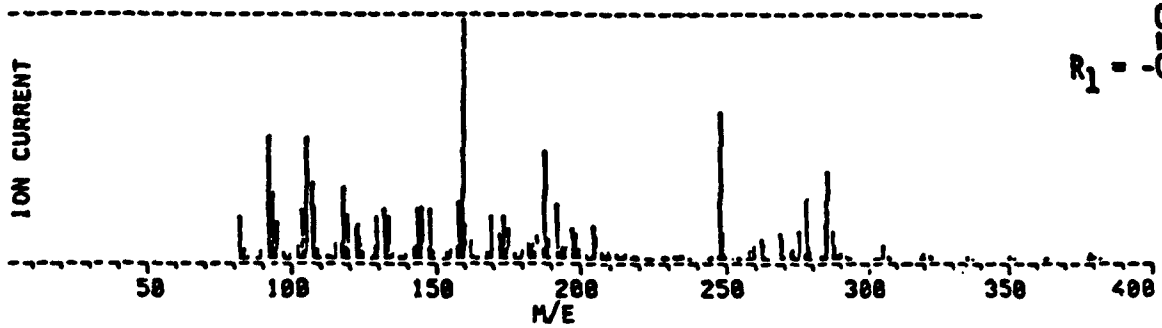


ELECTRON IMPACT

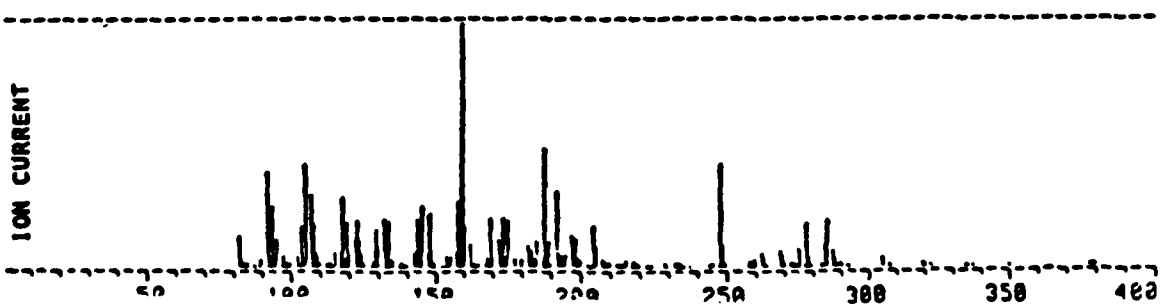


$R_1 = -\overset{O}{\overset{\|}{C}}-CH_3$ $R_2 = H$

C



15-MonoAc



Since epoxides are chemically reactive groups, we postulated that the 12, 13 epoxide moiety of anguidine may be hydrolized by epoxide hydrolase, or conjugated to glutathione with glutathione transferase. Anguidine was incubated with epoxide hydrolase from β-naphthoflavone induced microsomes (0.6 mg microsomal protein) in 50 mM Tris, pH 9.0 for 1 hour at 37° C with shaking. Metabolism was not observed by HPLC and scintillation counting. (The presence of epoxide hydrolase activity in the microsomes was determined with (7-^{3}H)-styrene glycol). Anguidine was incubated with 5 mM reduced glutathione and 0.01 IU glutathione transferase from rat liver cytosol in 10 mM K_2PO_4, pH 7.75, with shaking at 37° C for up to 18 hours. (Enzyme units were determined by Sigma using 1,2-epoxy-3-(p-nitrophenoxy)-propane as substrate). Again, metabolism was not observed by HPLC and scintillation counting. Therefore, the epoxide moiety does not appear to be metabolized in vitro.

A major route for detoxication of xenobiotics in mammalian systems is by conjugation to glucuronic acid in the presence of microsomal glucuronyl transferase. To determine if this is a route of detoxication for anguidine, 12 μM ^{3}H-anguidine, 6-9 mM UDP-glucuronic acid, and 0.6 mg microsomal protein from β-naphthoflavone induced microsomes was incubated with 2.5 mM $MgCl_2$ in 10 mM K_2PO_4 buffer at pH 7.7 for up to 3 hours at 37° C, with shaking. The incubation mixture was separated by reverse-phase HPLC and fractions were monitored by scintillation counting. A new peak was observed that did not appear in control incubations (Figure 8). This peak was also observed in incubations of anguidine or 15-monoacetoxyscirpenol with ^{14}C-UDP-glucuronic acid (data not shown). We were unable to hydrolyze this metabolite by incubation with either bovine liver or E. coli β-glucuronidase under high enzyme concentrations and long incubation times. This resistance to hydrolysis has important implications for detoxication in vivo.

The glucuronide was analyzed by Fast Atom Bombardment Mass Spectrometry. A MH+ peak is observed at m/e 501, corresponding to the mass expected for the glucuronide of a monoacetylated trichothecene (Figure 9). A peak due to the loss of the sugar is observed at m/e 307, and the loss of a sugar plus acetate group at m/e 265. The sample is prepared in glycerol, therefore, a number of glycerol (G) clusters are also observed.

The NMR spectrum of this peak (Figure 10) most closely matches that of 15-monoacetoxyscirpenol (Figure 11), but with a few changes. In addition to the proton resonances from the glucuronic acid (3.5-3.6 ppm and the C2 proton of the sugar in the β-configuration at 4.6 ppm), both the H3 and H4 resonances of the trichothecene have shifted downfield. Therefore, the glucuronic acid could be conjugated at either the C3 or C4 position. The position of conjugation is important. Anguidine and all of the hydrolysis products have an available hydroxyl group for conjugation at the C3 position, while that is not true of the C4 position (4-acetoxyscirpenediol has an acetate group in this position). Elimination of conjugated metabolites (increased polarity) by the kidney would be faster than that of unconjugated metabolites, decreasing the possibility of interaction with other macromolecules. By collaboration with Dr. Roush's group, we were able to identify the glucuronide as a conjugate at the C3 position (see annual summary report: W.R. Roush).

Figure 12 shows what we know about the in vitro metabolism of anguidine. Anguidine is rapidly metabolized to 15-monoacetoxyscirpenol, and to a lesser extent to 4-acetoxyscirpenediol and scirpenetriol. 15-Monoacetoxyscirpenol

FIGURE 8

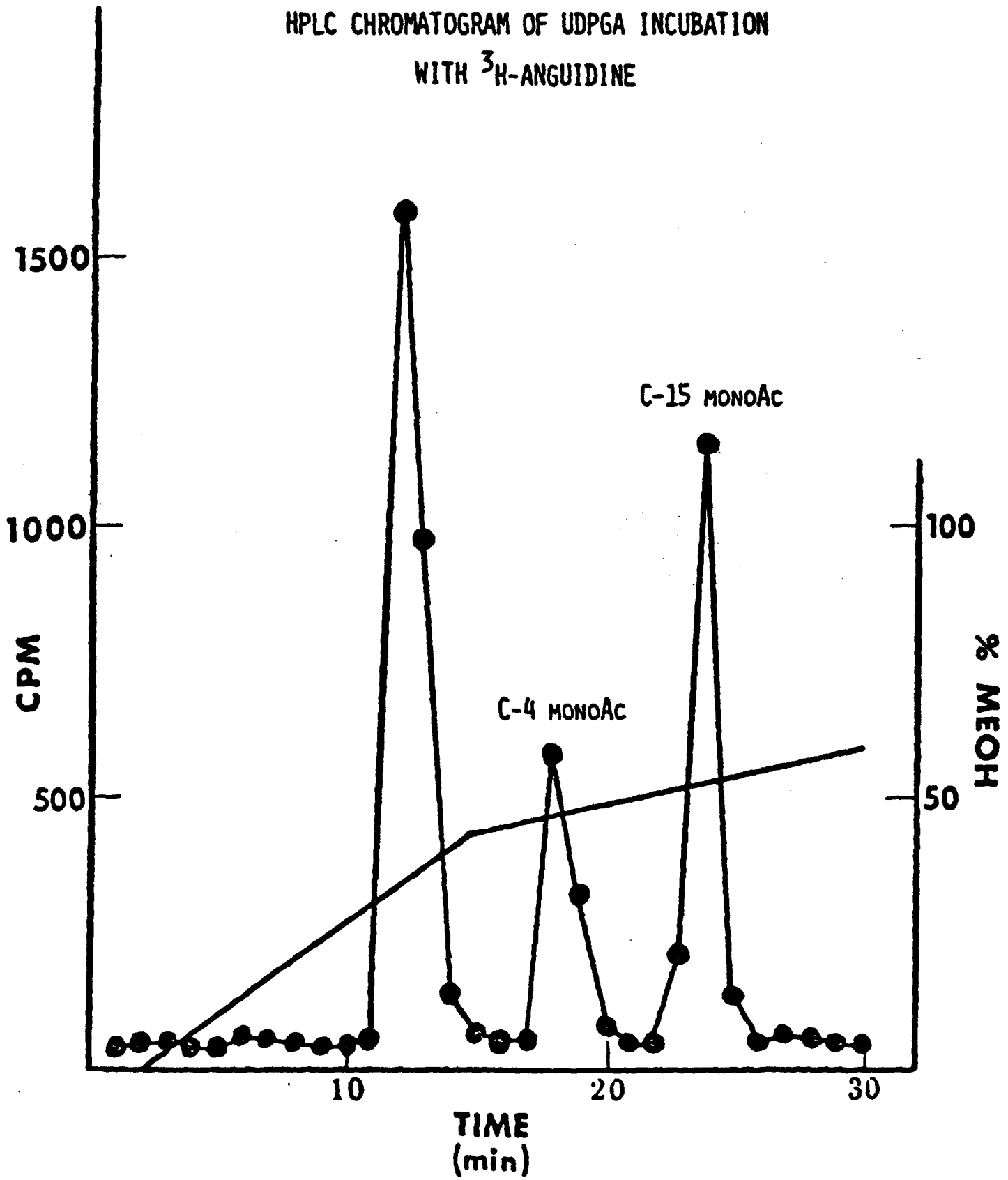

HPLC CHROMATOGRAM OF UDPGA INCUBATION
WITH ^{3}H-ANGUIDINE
1500
1000
500
CPM
C-15 MONOAC
C-4 MONOAC
100
50
% MEOH
10
20
30
TIME
(min)

FIGURE 9

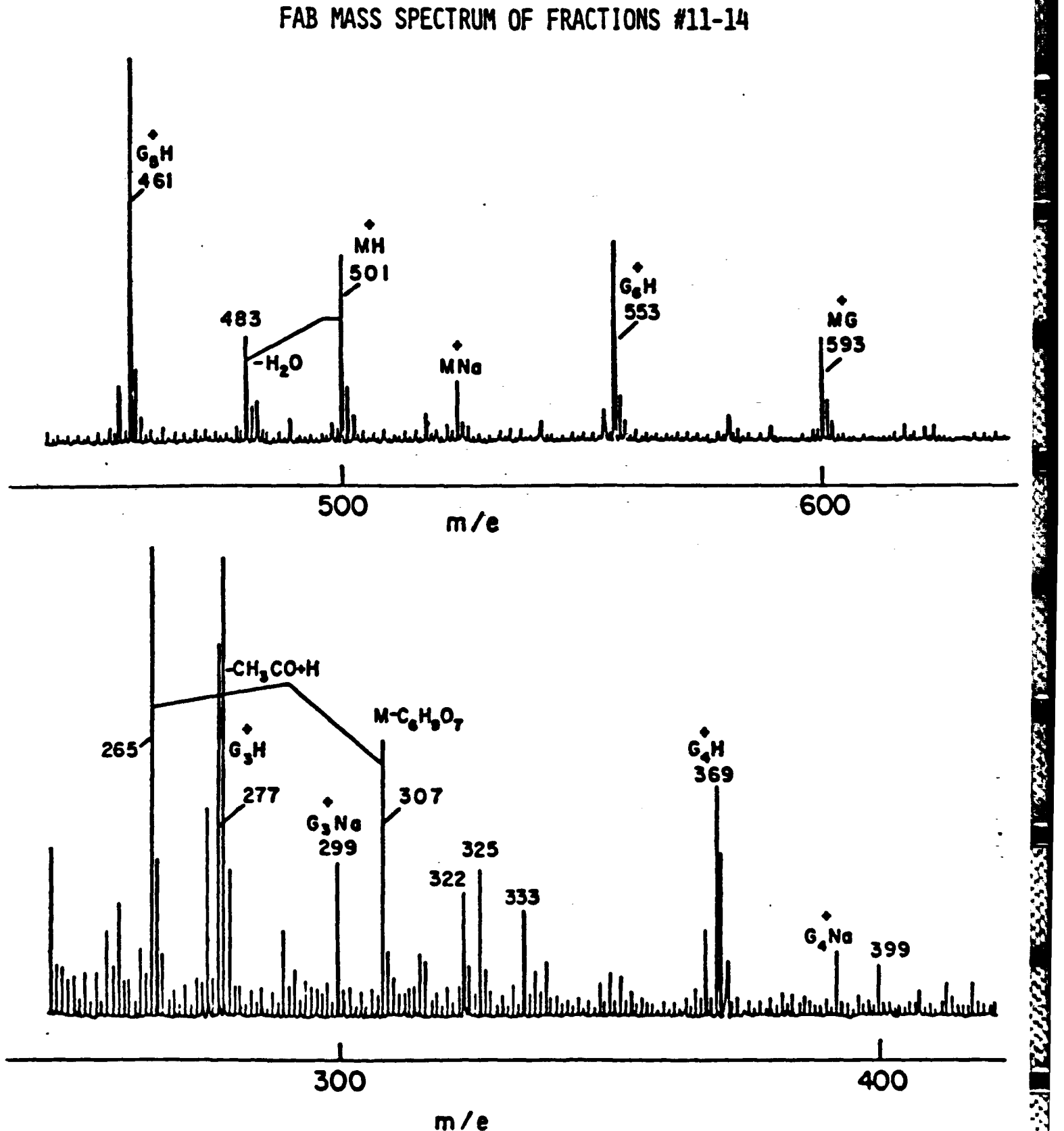

FAB MASS SPECTRUM OF FRACTIONS #11-14
G8H
461
MH
501
483
-H2O
MNa
G8H
553
MG
593
500
600
m/e
-CH3CO+H
M-C6H9O7
265
G3H
277
G3Na
299
307
325
322
333
G4H
369
G4Na
399
300
400
m/e

FIGURE 10

NMR OF FRACTIONS #11-14

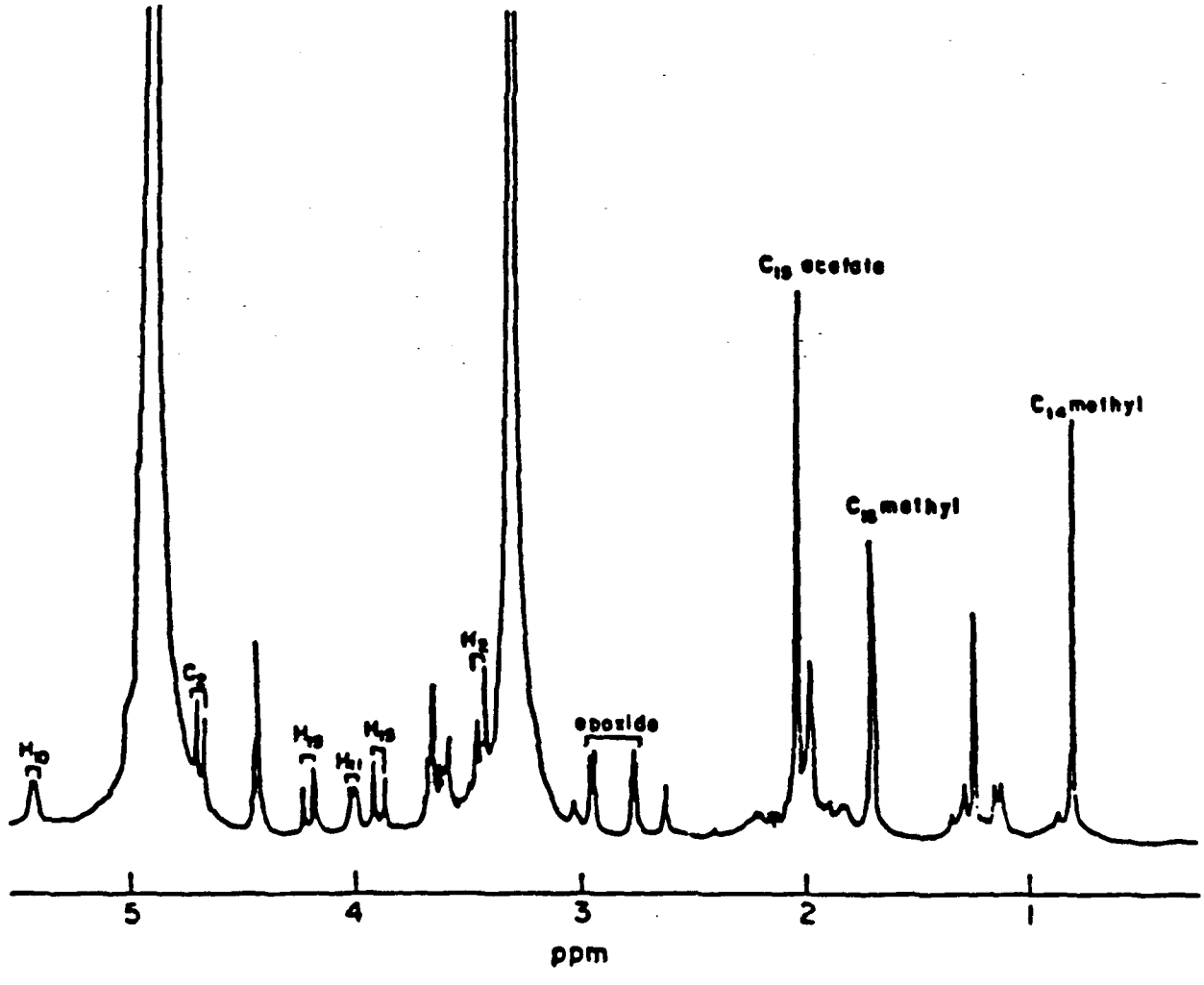


FIGURE 11

NMR OF SYNTHETIC C-15-MONOACETOXYSCIRPENOL

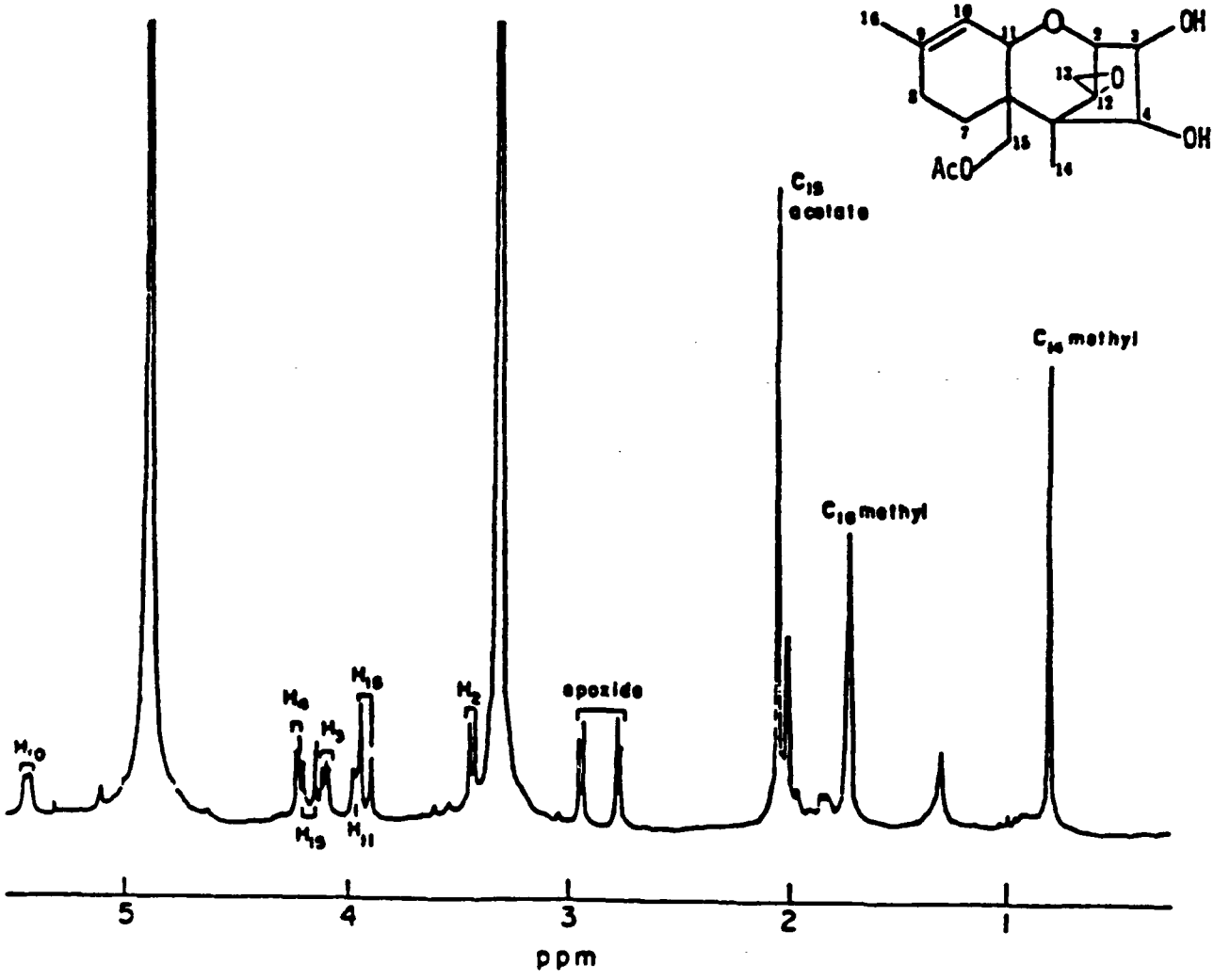
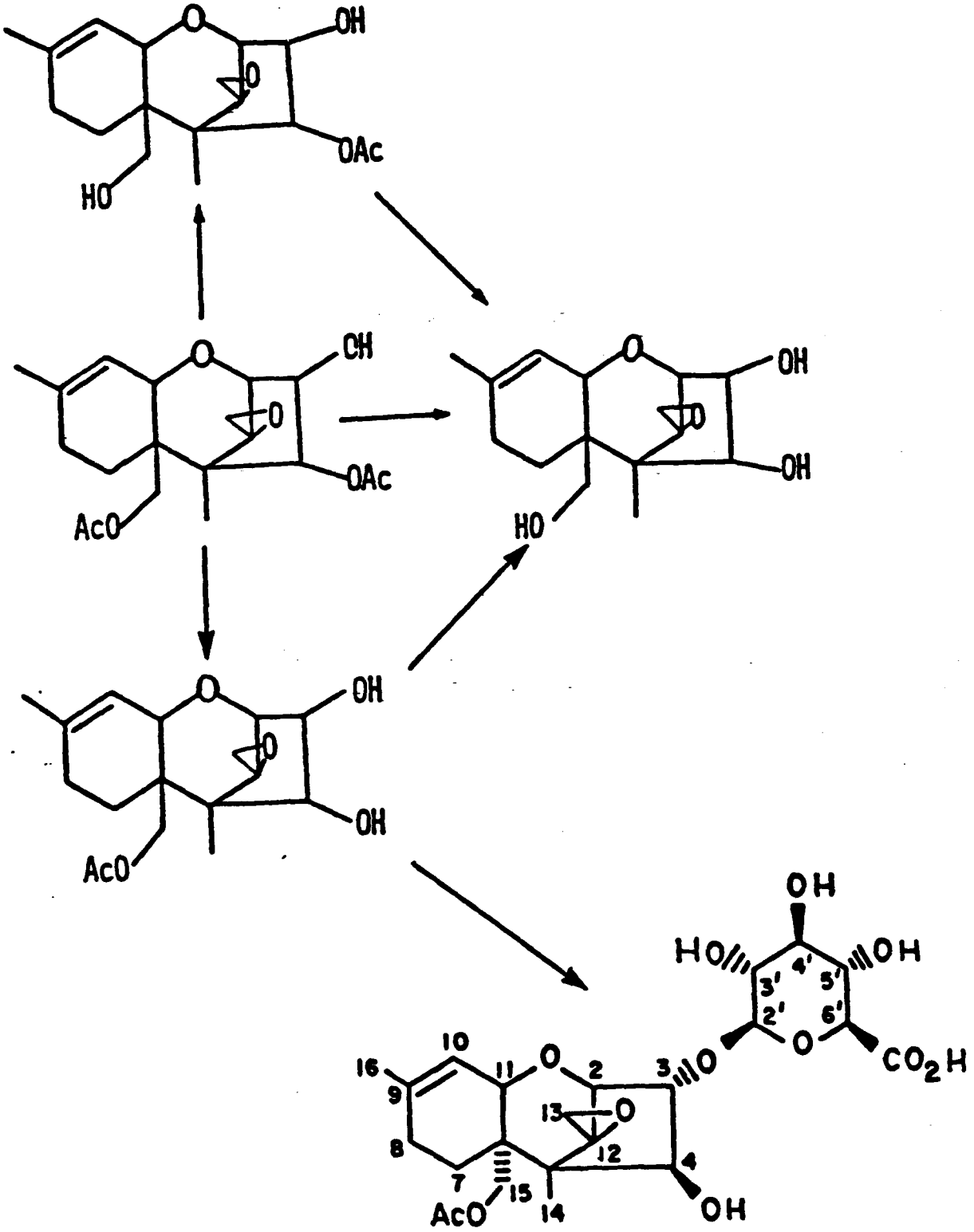


FIGURE 12

IN-VITRO METABOLISM OF ANGUIDINE (DAS)



can then conjugate to glucuronic acid at the C3 position.

Our in vitro work has recently been published in the J. Amer. Chem. Soc. 107: 3354-3355 (1985). That publication represents a succinct summary of the unambiguous structure proof of the glucuronide formed in vitro. At the end of the paper we mention our initial findings from our in vivo work. CD-1 mice were dosed by Dr. W.F. Busby with either [^{3}H]-DAS or unlabeled DAS and the urine from each treatment was collected for 48 hours after dosing. The urine was treated as shown on the scheme that follows. The HPLC chromatograms from step 5 (Figure 13) and step 8 (Figure 14) are shown. Those two figures indicate the previously determined retention times of the in vitro metabolites. Upon rechromatography under the same conditions the retention times change. At present we have determined that the major peak in Figure 13 which in the figure cochromatographs with the C15-monoacetate glucuronide has a FAB-mass spectrum consistent with a monoacetate glucuronide. However, when treated with limpet β-glucuronidase and then analyzed by HPLC the resulting product is the C4-monoacetate. The retention time is very close to that of the C15-monoacetate, C3-glucuronide so we feel that the in vivo product most likely will be the C4-monoacetate, C3-glucuronide but unambiguous structure proof has not been completed. Total analysis on all in vivo metabolites is under way.

We have tested the in vitro glucuronide (C15-monoacetate, C3-glucuronide) in a rabbit reticulocyte cell free protein synthesizing system and found it to be inactive at concentrations where anguidine totally inhibits protein synthesis. At least in this assay, then, this metabolite appears to be non-toxic. This coupled with our findings that this glucuronide is not hydrolyzed by E. coli or bovine liver β-glucuronidase continues to hold interest as a quantitatively important pathway of detoxication.

Other in vitro work includes studies with the purified glucuronyl transferase in order to determine substrate specificity. We found in microsomal experiments with anguidine that hydrolysis preceeds glucuronyl conjugation. The esterases are present in the microsomal fraction along with the transferase. Therefore, we purified the transferase and found the anguidine was not a substrate but the C15-monoacetate was. Future work will continue to probe the substrate specificity in an effort to understand this toxicologically significant pathway.

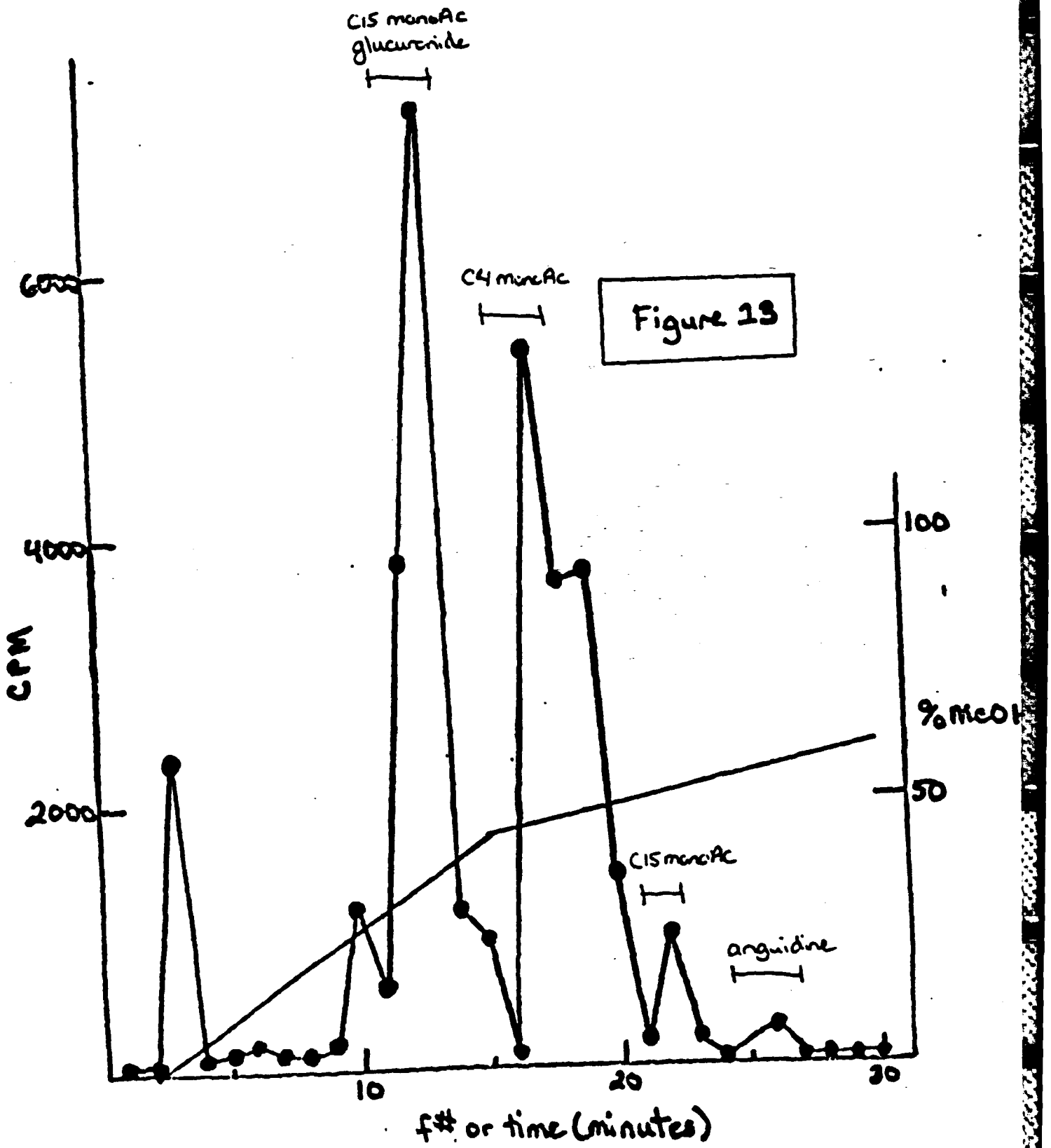
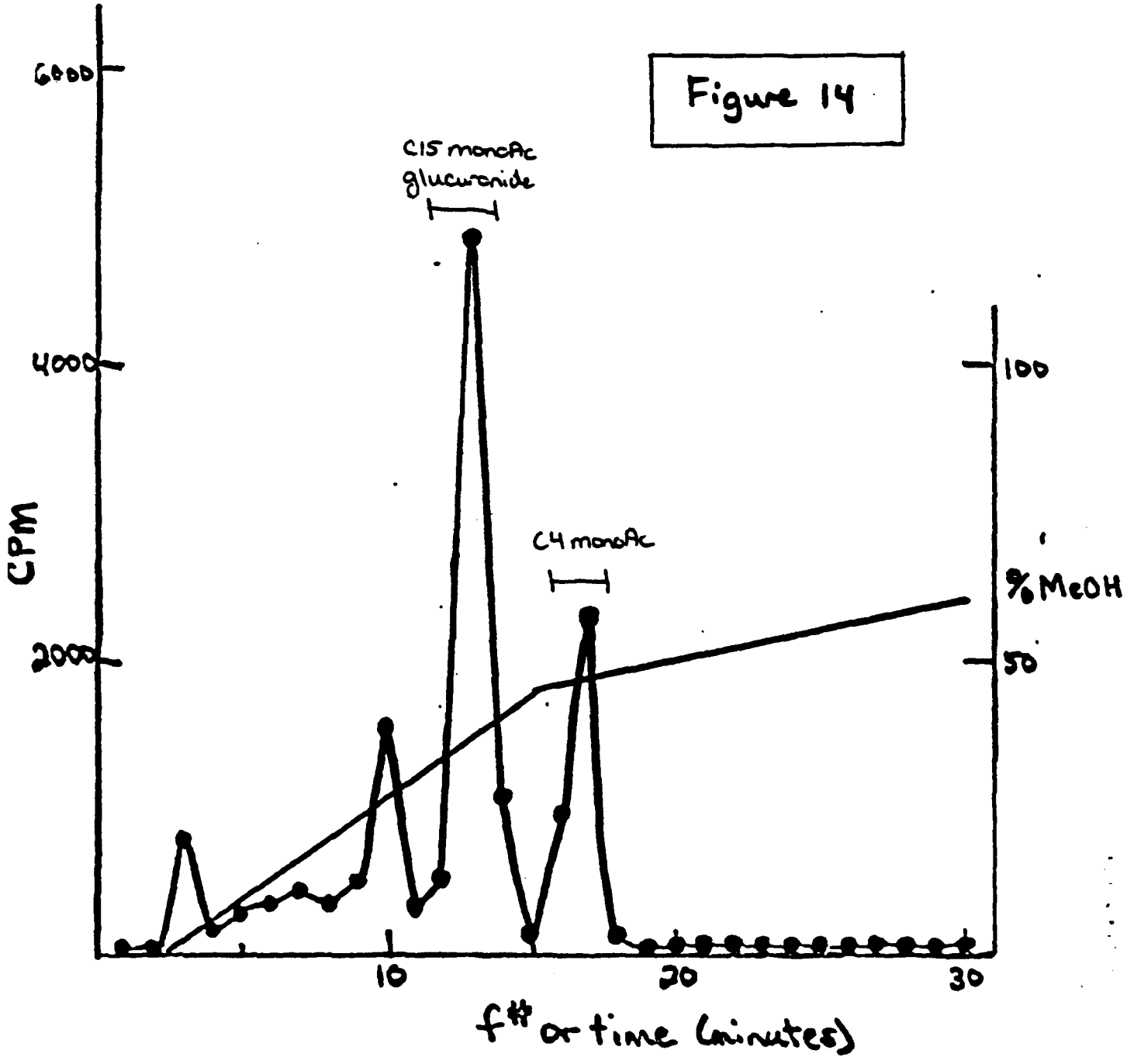

C15 monoAc glucuronide
C4 monoAc
C15 monoAc
anguidine
6000
4000
2000
CPM
100
50
%MeOH
10
20
30
f# or time (minutes)

Figure 13

Figure 14


6000
4000
2000
CPM
C15 monoAc
glucuronide
C4 monoAc
100
50
% MeOH
10
20
30
f# or time (minutes)

Segment 1B - *In Vivo* Metabolism

G.N. Wogan
W.F. Busby, Jr.

SEGMENT IB
G.N. Wogan
W.F. Busby Jr.

1) Introduction

The primary objective of this segment has been to measure response in rats and mice to topically applied anguidine. The first phase of this research effort involved: (1) the development of techniques to measure topical absorption *in vivo* by construction of non-occlusive skin patches suitable for use in different animal species and application site areas and (2), the verification of patch integrity to ensure against violation of the site by the animal and/or loss of the anguidine from the site and possible subsequent exposure of the animal by routes other than dermal absorption.

Once suitable methods were developed, the investigations progressed along two lines of inquiry. The first effort is a collaborative study (discussed further under Segment 3) to examine the toxicity of topically absorbed anguidine in rats and mice by monitoring the time-course of the histopathologic response in various tissues. Secondly, the skin patch technique is being applied to study patterns of tissue distribution and excretion of topically absorbed [^{3}H]-anguidine in rats and mice.

2) Skin Patch Construction and Integrity Verification

Figure 1 depicts the essential details of the construction and application of a typical non-occlusive skin patch. The experimental animal was carefully shaved around the mid-section with electric clippers under anesthaesia and an elastic adhesive bandage, with an opening cut to expose the application site and the surrounding area, was secured. The skin patch was a single unit constructed of 260 mesh nylon screen and aluminum screening which were separated by an adhesive foam spacer held together by strips of elastic adhesive bandage. The patch was placed over the application site and fastened to the mid-section bandage on all sides by additional bandage strips (not shown). The anterior and posterior bandage strips encircled the animal and were the last ones to be attached.

The integrity of the skin patch was measured in rats and mice by using [^{14}C]-Direct Black 19 dye (DB19) as an indicator. This dye, even if it did penetrate skin, was not excreted in urine or feces of the rat, mouse, or rabbit, hence could be used to monitor skin patch integrity by measuring radiolabel in urine, feces, and cage washings.

The results from patch integrity experiments with [^{14}C]-DB19 in the rat and mouse are shown in Table 1. The rat studies were performed with 275 g animals with a 4 cm^2 application site, which represented conditions used in the earliest anguidine toxicity experiments. Clearly, only very low levels of activity (0.03% of the applied dose) were detected in excreta and cage washings of these animals. Current studies of both anguidine toxicity and toxicokinetics now employ 100 g rats (with a 1.44 cm^2 application site) to conform to the age of the animals used in Segment 3. Validation measurements of patch integrity with these animals (not shown) gave essentially equivalent results.

Data from mice also showed very low levels of activity in urine and cage washings but somewhat higher levels in feces (0.26% of the applied dose). This was due to minor leakage or violation of the skin patches by two of the animals toward the end of these early experiments. Because of their small size and higher level of physical activity, it was more difficult to apply and maintain the patches on mice. However, with the additional experience that has been gained, we are confident that no significant loss of anguidine is occurring in

FIGURE 1

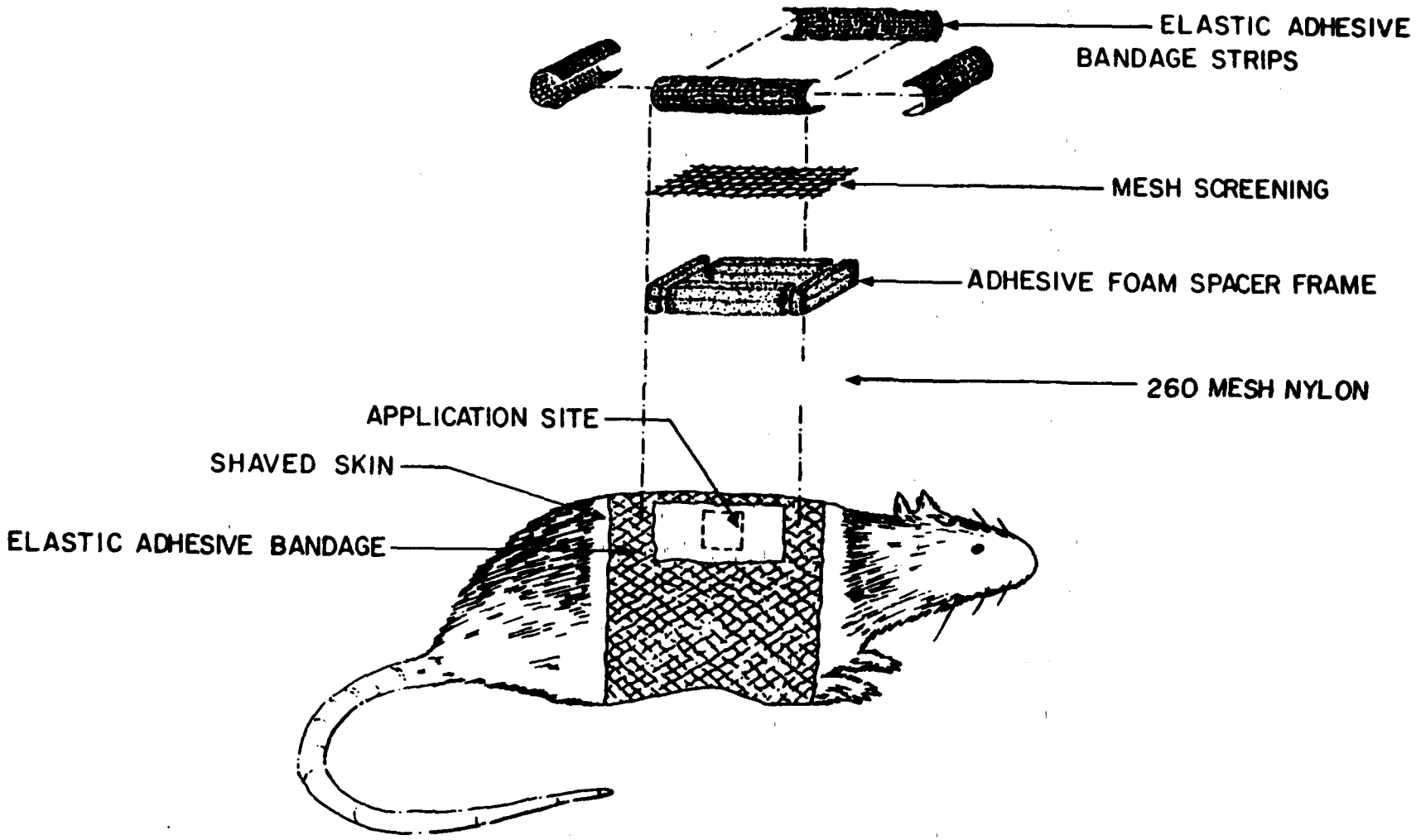

ELASTIC ADHESIVE BANDAGE STRIPS
MESH SCREENING
ADHESIVE FOAM SPACER FRAME
260 MESH NYLON
APPLICATION SITE
SHAVED SKIN
ELASTIC ADHESIVE BANDAGE

TABLE 1

LOSS OF [^{14}C]-DB19 DYE FROM SKIN APPLICATION SITE

Time (hours)	Rat[a,c] (% dose - cumulative)		Mouse[b,c] (% dose - cumulative)	
	Urine and cage wash	Feces	Urine and cage wash	Feces
24	0.01 ± 0	0	0.02 ± < 0.01	0
48	0.02 ± <0.01	0	0.02 ± < 0.01	0
72	0.02 ± <0.01	0	0.02 ± < 0.01	0.01 ± 0
96	0.02 ± <0.01	0	0.03 ± < 0.01	0.16 ± 0.15
120	0.03 ± <0.01	0	--	--
144	0.03 ± <0.01	0	--	--
168	--	--	0.03 ± < 0.01	0.26 ± 0.14

[a] 1.85×10^7 DPM applied on 2 cm x 2 cm application site.

[b] 1.96×10^6 DPM applied on 0.65 cm x 0.65 cm application site.

[c] Data expressed as the Mean ± S.E. from 4 animals in each group.

the experiments now in progress.

Table 2 summarizes the parameters used in topical absorption studies of both anguidine toxicity (Segment 3) and [^{3}H]-anguidine tissue distribution and excretion. Two sizes of skin patch were used with the mouse. The "small patch" parameters are proportional on a body weight basis to the dose and application site area used with the rat. Under these conditions of topical exposure approximately one-half of the rats died within the first 3 days, but the mice survived without obvious clinical signs of toxicity. The "large patch" was initially developed to maximize the topical dose of anguidine in an attempt to induce toxicity in this species. Mice treated with this 2.5-fold higher dose of anguidine also appeared clinically refractory. This clearly established the insensitivity of the mouse to the toxin relative to the rat and complemented the difference in toxicity also observed with i.v. and i.p. treatment (Sato and Ueno, 1977; National Research Council, 1983).

3) Excretion and Tissue Distribution of Topically Applied [^{3}H]-Anguidine

These data describe 7-day topical absorption studies of the tissue distribution and excretion of [^{3}H]-anguidine residues using the application site parameters summarized in Table 2 for the rat and the mouse (small patch). The [^{3}H]-anguidine dose, however, is proportionally reduced to avoid toxicity in the rat and to minimize waste of the radiolabeled compound. The volume of DMSO vehicle applied to the skin remains as stated.

a) Rat

[^{3}H]-anguidine (sp. act.: 13 uCi/umole) was exchanged into DMSO and 35 ul (0.1 mg, 3.5 uCi) was topically applied to the shaved dorsum of 90-110 g male Fischer rats. A non-occlusive skin patch was applied to the site as previously described and the animals were maintained in metabolic cages for 7 days. Urine and feces were collected over the course of the experiment and the organs, carcass, application site skin, and skin patch were saved for processing and measurement of radioactivity.

Excellent recovery of the radioactivity applied to the skin was obtained for each of the 4 rats with a mean of over 97% and a range of 95-99% (Table 3).

The cumulative excretion of radiolabel in urine and feces is shown in Table 4. Approximately 45% of the total applied dose was excreted during the experiment. About two-thirds of this amount was measured in urine and the remainder was present in feces. Most of the excretion of radiolabel (over 70%) occurred within the first 24 hr.

A detailed analysis of the distribution of [^{3}H]-anguidine residues in organs, GI tract contents, carcass, application site skin, and skin patch and bandages are listed in Table 3. Excluding the application site skin, approximately 1% of the applied radioactivity was present within the organs (0.3%), GI tract contents (0.2%), and carcass (0.5%). No exceptional accumulation in specific organs was noted, although the liver, femurs, and small intestine appeared to have elevated levels of radioactivity. The relatively high levels in "miscellaneous organs" (which includes a substantial amount of blood) and in the carcass suggest that significant radiolabel may be associated with blood cells, since essentially no label was detected in blood plasma.

Nearly 18% of the applied dose was found in the skin taken from the application site. Simple acetone extraction removed only about 45% of this amount, suggesting that most of this radiolabel was not just superficially layered on the skin surface.

There were considerable differences in the amount of [^{3}H]-anguidine residues recoverable from the skin patch and bandages. It is this variation that largely accounts for the range of results obtained with the excretion data shown in

TABLE 2

PARAMETERS OF ANGUIDINE PERCUTANEOUS ABSORPTION EXPERIMENTS

Animal	Avg. Body Wt. (g)	Anguidine Dose (mg)	DMSO (@1)	Application Site Size (cm)	Application Site Area (cm^2)	Body Surface Area (cm^2)[a]
RAT	100	2.625[b]	35	1.2 x 1.2	1.44[b]	205.7
MOUSE (small patch)	27.5	0.75[b]	10	0.65 x 0.65	0.42[b]	86.6
MOUSE (large patch)	27.5	1.875	25	1.0 x 1.0	1.0	86.6

[a] Calculated from $A = KW^{2/3}$, where W = body wt (g) and K = constant (mouse, 9.5; rat, 9.6).

[b] Anguidine dose and application site area is approximately proportional to body weight.

TABLE 3

DISTRIBUTION OF RADIOLABEL FROM TOPICALLY APPLIED [^{3}H]-ANGUIDINE

TISSUE OR MATERIAL	% OF DOSE[a] RAT	MOUSE
A. INTERNAL ORGANS		
stomach	0.007 ± .002	0.011 ± .002
small intestine (A)	0.019 ± .003	0.028 ± .008
" (B)	0.012 ± .003	0.020 ± .006
" (C)	0.008 ± .003	0.010 ± .004
cecum	0.008 ± .004	0.014 ± .005
large intestine	0.009 ± .003	0.009 ± .003
lungs	0.008 ± .002	0.014 ± .008
heart	0.008 ± .003	0.009 ± .005
liver	0.029 ± .010	0.013 ± .010
spleen	0.009 ± .002	0.006 ± .002
bladder	0.003 ± .001	0.008 ± .003
kidney	0.008 ± .003	0.005 ± .004
testes	0.009 ± .002	0.010 ± .005
brain	0.009 ± .002	0.006 ± .002
blood plasma (aliquot)	0.003 ± .001	--
misc. organs	0.157 ± .068	--
abdominal fat	0	0.011 ± .003
SUB-TOTAL	0.305 ± .086	--
B. GI TRACT CONTENTS		
stomach	0.013 ± .003	0.001 ± .001
small intestine (A)	0.015 ± .003	0.001 ± .001
" (B)	0.018 ± .005	0.001 ± .001
" (C)	0.027 ± .005	0
cecum	0.080 ± .028	0
large intestine	0.031 ± .003	0
SUB-TOTAL	0.184 ± .034	0.003 ± .001

TABLE 3 (cont.)

TISSUE OR MATERIAL	% OF DOSE RAT	MOUSE
C. CARCASS AND SKIN[b]		
femurs	0.024 ± .012	0.016 ± .005
skin - acetone	0.042 ± .015	--
skin - water	0.101 ± .021	--
carcass - acetone	0.137 ± .037	--
carcass - water	0.162 ± .031	--
SUB-TOTAL	0.465 ± .066	
D. EXCRETA		
urine	30.421 ± 7.354	7.107 ± 2.133
feces	15.090 ± 4.853	1.917 ± .467
SUB-TOTAL	45.511 ± 11.414	9.024 ± 2.460
E. APPLICATION SITE		
skin - acetone	7.779 ± 3.050	
skin - water	10.330 ± 2.334	
nylon mesh - acetone	4.477 ± 1.708	
nylon mesh - water	1.060 ± 0.254	
bandages and patch - acetone	6.170 ± 3.739	
bandages and patch - water	21.166 ± 14.057	
SUB-TOTAL	50.954 ± 12.088	
TOTAL RECOVERY	97.420 ± 0.863	

[a] Mean ± S.E. in 4 animals of each species.
[b] Excluding application site skin.

Table 4. It seems probable that in some cases, the liquid DMSO vehicle may be wicked into parts of the skin patch other than the overlying nylon mesh. Future experiments will employ a reduced volume of the DMSO vehicle to eliminate this source of variation.

b) Mouse

These experiments were performed in a manner similar to those using rats except that 1 uCi [^{3}H]-anguidine (0.03 mg) was applied to the shaved dorsum of 25-30 g male CD-1 mice (0.65 cm x 0.65 cm application site).

The total cumulative excretion of radiolabel for the mouse over the 7-day period was 9% of the dose applied to the skin, or only one-fifth of the amount excreted by the rat (Table 4). Nearly 80% of the amount excreted appeared in the urine compared to only 66% in the rat. The time-course kinetics for excretion in both the rat and mouse were very similar in that 70% of the radiolabel appeared in the urine and feces within the first 24 hr in both cases.

The final compilation of the distribution data for the mouse experiment is not yet completed (Table 3) but there is sufficient information to indicate no major reservoir of [^{3}H]-anguidine residues in either the rat or the mouse. In both species there seems to be an increase in the amount of radiolabel present in the tissue of the small intestine and in the femurs. It is also apparent that there is little or no radiolabel associated with the contents of the gastrointestinal tract in the mouse, presumably reflecting the much lower levels of fecal excretion.

It appears, on the basis of the results obtained to date, that the relative insensitivity of the mouse to topically applied anguidine may be due in large part to reduced permeability of mouse skin and/or greater sequestering of the toxin within the skin at the application site.

References

1) National Research Council. "Protection Against Trichothecene Mycotoxins," pp. 93-157, National Academy Press, Washington, 1983.

2) Sato, N. and Ueno, Y. In "Mycotoxins in Human and Animal Health," (J.V. Rodricks, C.W. Hesseltine, and M.A. Mehlman, Eds.), pp. 295-307, Pathotox Publishers, Park Forest South, Ill., 1977.

TABLE 4

CUMULATIVE EXCRETION OF RADIOLABEL FROM TOPICALLY APPLIED [^{3}H]-ANGUIDINE

TIME (hr)	% TOTAL DOSE EXCRETED[a] RAT URINE	RAT FECES	RAT TOTAL	MOUSE URINE	MOUSE FECES	MOUSE TOTAL
24	22.77 ± 5.27	8.99 ± 2.76	31.76 ± 7.43	4.96 ± 1.69	1.28 ± 0.32	6.24 ± 1.77
48	25.85 ± 6.02	12.40 ± 3.98	38.25 ± 9.24	6.16 ± 1.97	1.53 ± 0.38	7.70 ± 2.12
72	27.90 ± 6.57	13.45 ± 4.36	41.35 ± 10.18	6.59 ± 2.03	1.68 ± 0.41	8.27 ± 2.25
96	29.13 ± 6.98	14.13 ± 4.52	43.26 ± 10.76	6.82 ± 2.09	1.80 ± 0.44	8.62 ± 2.36
168	30.42 ± 7.35	15.09 ± 4.85	45.51 ± 11.41	7.11 ± 2.13	1.92 ± 0.47	9.02 ± 2.46

[a] Mean ± S.E. from 4 animals of each species.

Segment 2A

Chemistry and Synthesis of Compounds as Protective Agents

G. Buchi and G. Lee

Segment 2A - Chemistry
G. Buchi and G. Lee

As noted in background and proposed research areas described above, it has been the aim of this segment to synthesize materials for testing in animal systems which might prove to be effective in preventing or alleviating the effects of trichothecene toxins. An initial attempt was made to prepare materials that might react with the internal epoxide of the trichothecene molecule. These attempts were not successful and this approach was discontinued during the first year of the contract.

Our efforts were then directed toward a group of compounds including thiazolidines and selenium-containing analogues.

According to Meister et al., L-2-oxothiazolidine-4-carboxylate (1), which is converted to L-cysteine by the enzyme r-oxo-L-prolinase, promotes the synthesis of glutathione and appear to protect the organism from certain toxic compounds.

We have prepared substantial quantities of 1 in crystalline form and submitted the material to Professors Newberne and Rogers for testing. Initial experiments indicated that this thiazolidine did not protect mice against anguidine injury. Compound 1 is highly insoluble in aqueous media and it was decided to prepare the sodium salt which, as anticipated, was found to be water soluble. This sodium salt, unfortunately, has thus far resisted crystallization but the "syrup" is stable at room temperature, and the Newberne-Rogers group reports that it does indeed reduce the toxicity of anguidine.

L-2-oxothiazolidine-4-carboxylate (1), promotes the synthesis of glutathione which in turn protects organisms against certain toxic compounds. Recent work by Newberne and Rogers suggests that compounds 1 reduces the toxicity of anguidine. Meister has already prepared a number of analogues of the oxothiazolidine 1 and tested their ability to serve as substrates for the enzyme 5-oxo-L-prolinase.

We have prepared the additional analogues 3-5 which seem to be unknown, and submitted them to Professor Newberne and Rogers for biological evaluation. There were only small amounts and the bioassays have not been completed.

We completed initial work on a synthesis of the selenium compound 2 which, however, was not completed at the time the contract terminated.

Segment 2B - Chemistry

W. Roush

Table I. Papers Published or Submitted for Publication Resulting from Studies Supported by this Contract

1. W.R. Roush, M.A. Marletta, S. Russo-Rodriguez, and J. Recchia, "Trichothecene Metabolism Studies: Isolation and Structure Determination of 15-Acetyl-3α-(1'β-D-glucopyranosiduronyl)-scirpen-3,4β,15-triol", J. Am. Chem. Soc. 1985, 107, 3354.

2. W.R. Roush and S. Russo-Rodriguez, "Synthesis of 4β-Acetoxy-scirpen-3α,15-triol", J. Org. Chem., in press.

3. W.R. Roush, M.A. Marletta, S. Russo-Rodriguez, and J. Recchia, "Trichothecene Metabolism Studies. 2. Structure of 3α-(1"β-D-Glucopyranosiduronyl)-8α-isovaleryloxyscirpen-3,4β,15-triol 15-Acetate Produced from T-2 Toxin In Vitro", Tetrahedron Lett., in press.

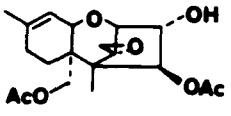
4. W.R. Roush and S. Russo-Rodriguez, "Trichothecene Degradation Studies: Synthesis of 12,13-Deoxyanguidine and 12,13-Deoxyverrucarol", J. Org. Chem., submitted.

This Progress Report summarizes research performed under the Mycotoxin Contract that terminated August 1, 1985. Our work has concentrated on the structure determination and synthesis of metabolites of anguidine and T-2 toxin, synthesis of ^{14}C-labelled anguidine for metabolism studies, and the development of a preparative route to nivalenol from anguidine. Work on nivalenol has been postponed pending completion of the anguidine and T-2 studies. Accomplishments in these areas are summarized below. Four papers describing portions of this work have been published or submitted for publication (see Table 1).

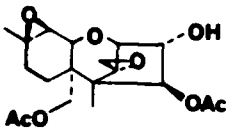
1. Characterization of the In Vitro Generated Metabolites of Anguidine

Prior to the initiation of our work in collaboration with Dr. Marletta, relatively little work on the metabolism of the trichothecenes had been reported.[1,2] T-2 toxin and 4-acetoxynivalenol had been examined in a number of organisms (broiler chicken, mice, rats, cows, etc.).[2] Anguidine, which has gone to Phase II clinical trials as an anticancer agent,[3] had been examined in microorganisms[4] and in dogs and monkeys,[5] the latter only for toxic manifestations. The only transformations documented prior to the initiation of our studies had been deacylation reactions of the trichothecene esters, which are promoted by microsomal esterases,[2c,6] and the oxidation of T-2 and HT-2 toxin *in vivo* and *in vitro* experiments leading to 3'-hydroxyl derivatives.[2a,b] The latter suggests the involvement of cytochrome P_{450} in the metabolism of T-toxin.

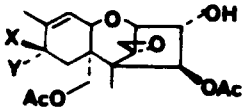
Initial efforts focused on the generation of a library of putative metabolites (e.g., 2-6) which might be obtained by cytochrome P_{450} oxidation of anguidine.[7] Compounds 2-4 were prepared according to literature procedures; 5 is a minor product of the SeO_2 oxidation of



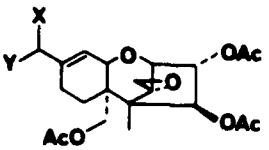
Anguidine (1)



(2)



3, X=H, Y=OH
4, X=Y=O

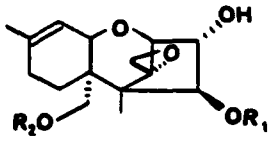


5, X=OH, Y=H
6, X=Y=O

3,4,15-scirpenetriacetate, and 6 was prepared in 47% yield by CrO_3-pyridine oxidation of the same substrate. Since Dr. Marletta found no evidence for P_{450}-mediated oxidation of anguidine in *in vitro*

experiments, however, our efforts to prepare additional "putative P_{450}-metabolites" were discontinued.

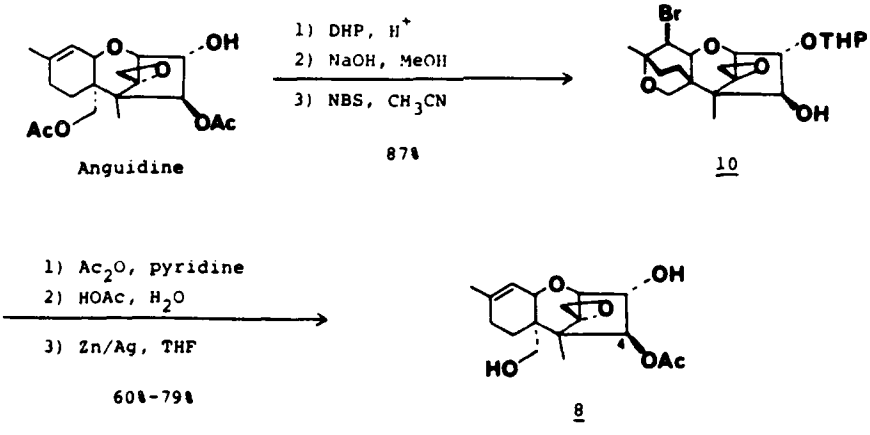
The first metabolites positively identified in <u>in vitro</u> experiments performed by Drs. Marletta and Recchia were 15-acetoxyscirpendiol (7), 4-acetoxyscirpendiol (8), and scirpentriol (9). These identifications were made by comparison with authentic samples provided by us.



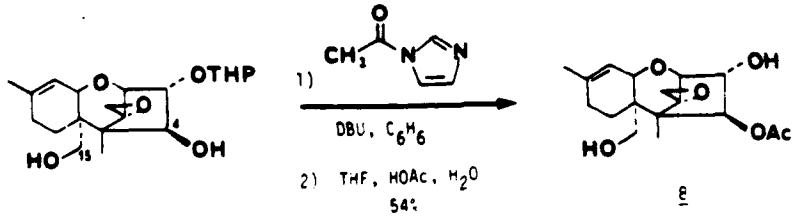
1 $R_1 = R_2 = Ac$ (anguidine)
7 $R_1 = H$, $R_2 = Ac$
8 $R_1 = Ac$, $R_2 = H$
9 $R_1 = R_2 = H$

Authentic samples of 15-monoacetate 7 and scirpentriol 9 were synthesized by literature procedures.[8] 4-Monoacetate 8 was synthesized initially by the route summarized in Scheme I. This route was developed since acylation of scirpentriol is not selective for C.4 (and, in fact, gives complicated product mixtures).[7] In addition, the reported method for preparation of 8 by selective hydrolysis of anguidine gave, in our hands, an apotrichothecene rather than 8.[8a] As an added dividend, it is noteworthy that Scheme I provides intermediates useful in our work on the synthesis of ^{14}C-labelled anguidine. A more direct synthesis of 8 developed subsequently is summarized in Scheme II.

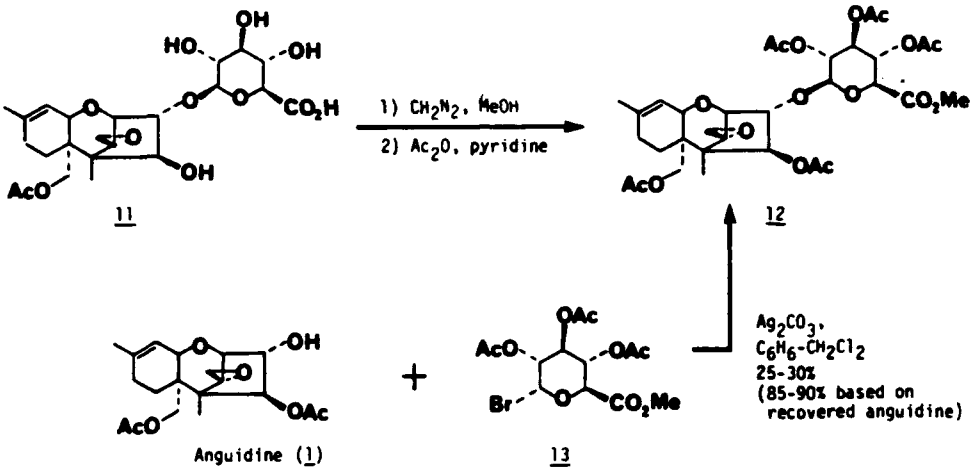
Scheme I



Scheme II



Marletta and Recchia isolated a 15-acetoxyscirpentriol-glucuronic acid conjugate from *in vitro* metabolism studies of anguidine. We established that **11** is the structure of this metabolite using the procedures described below. Treatment of a sample of **11** provided by Dr. Recchia with CH_2N_2 in methanol followed by acetic anhydride/pyridine afforded derivative **12** which was purified by chromatography. This compound proved to be identical in all respects to an authentic sample synthesized unambiguously from anguidine (**1**) and bromosugar **13** by a Koenigs-Knorr reaction (25-30% yield).[9] The 250-MHz ^1H spectra of synthetic and naturally-derived **12** are provided in Figures 1 and 2.



11 **12** **13**

This experiment rigorously establishes that the glucuronic acid residue is attached to C.3 of the trichothecene nucleus and that the glycosidic linkage is β. That the acetate residue in **11** is attached to C.15, and not C.4, was confirmed by NMR methods. The 250 MHz ^1H NMR spectrum of **11** appears in Figure 3.

This initial study suggested that glucuronidation may be an important pathway for trichothecene metabolism *in vivo*. Accordingly, Marletta and Recchia have studied the metabolism of anguidine in mice and have isolated two glucuronides as the major metabolites. Interestingly, neither of these metabolites is **11**. We are collaborating with Dr. Marletta on the structure determination of these compounds and are confident that complete assignments can be made in the coming year.

2. Characterization of an In Vitro Generated Glucuronic Acid Conjugate of T-2 Toxin

Drs. Marletta and Recchia have isolated a glucuronide from *in vitro* incubation of T-2 toxin with UDP-glucuronic acid and rat liver microsomes. ^1H NMR and FAB mass spectral analyses of the conjugate indicated that it is a glucuronide derivative of HT-2 toxin. Following the successful strategy used previously in the anguidine studies, we

Figure 1 250 MHz ^1H NMR Spectrum of Synthetic 12

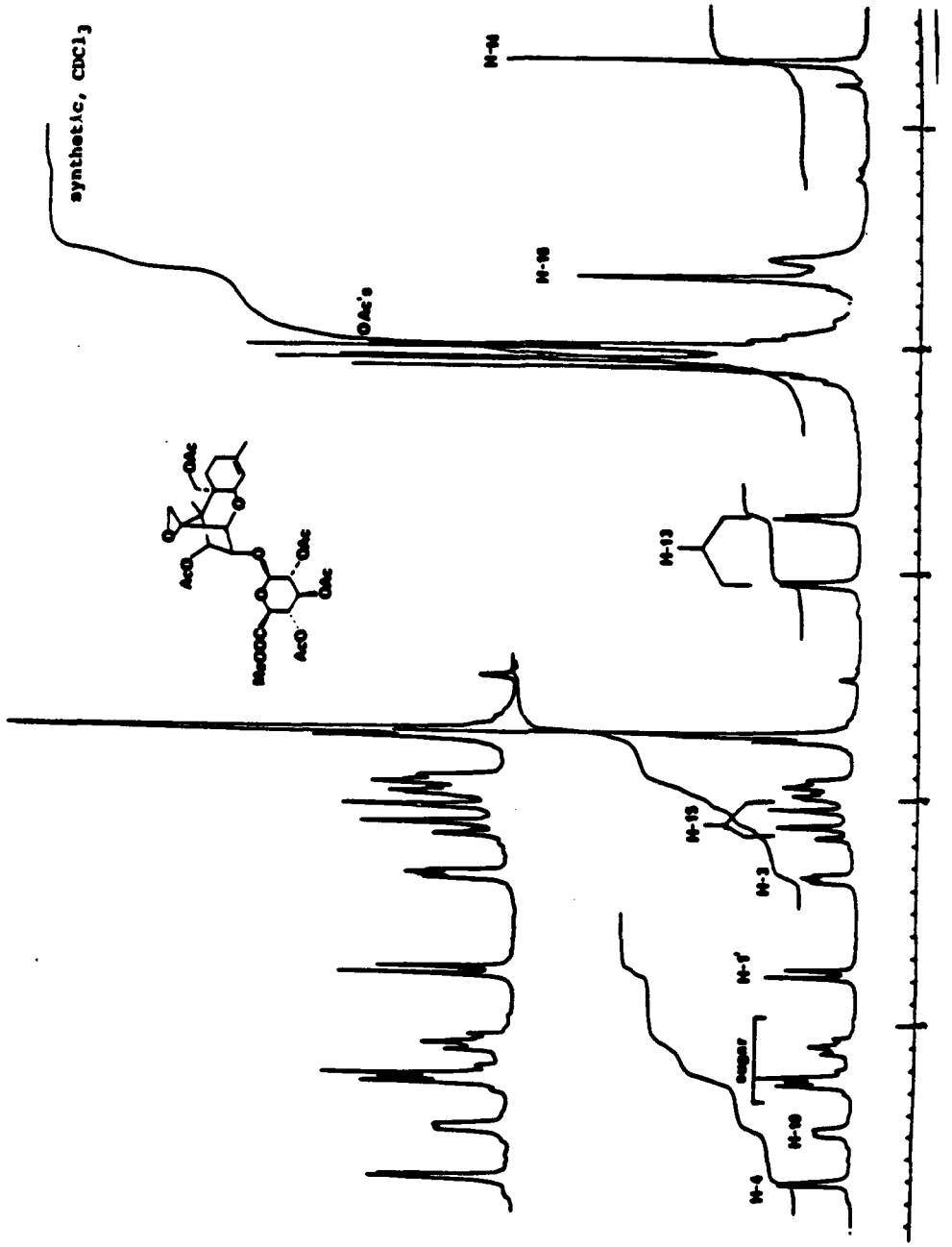


Figure 2 250 MHz ^1H NMR Spectrum of Naturally-Derived 12

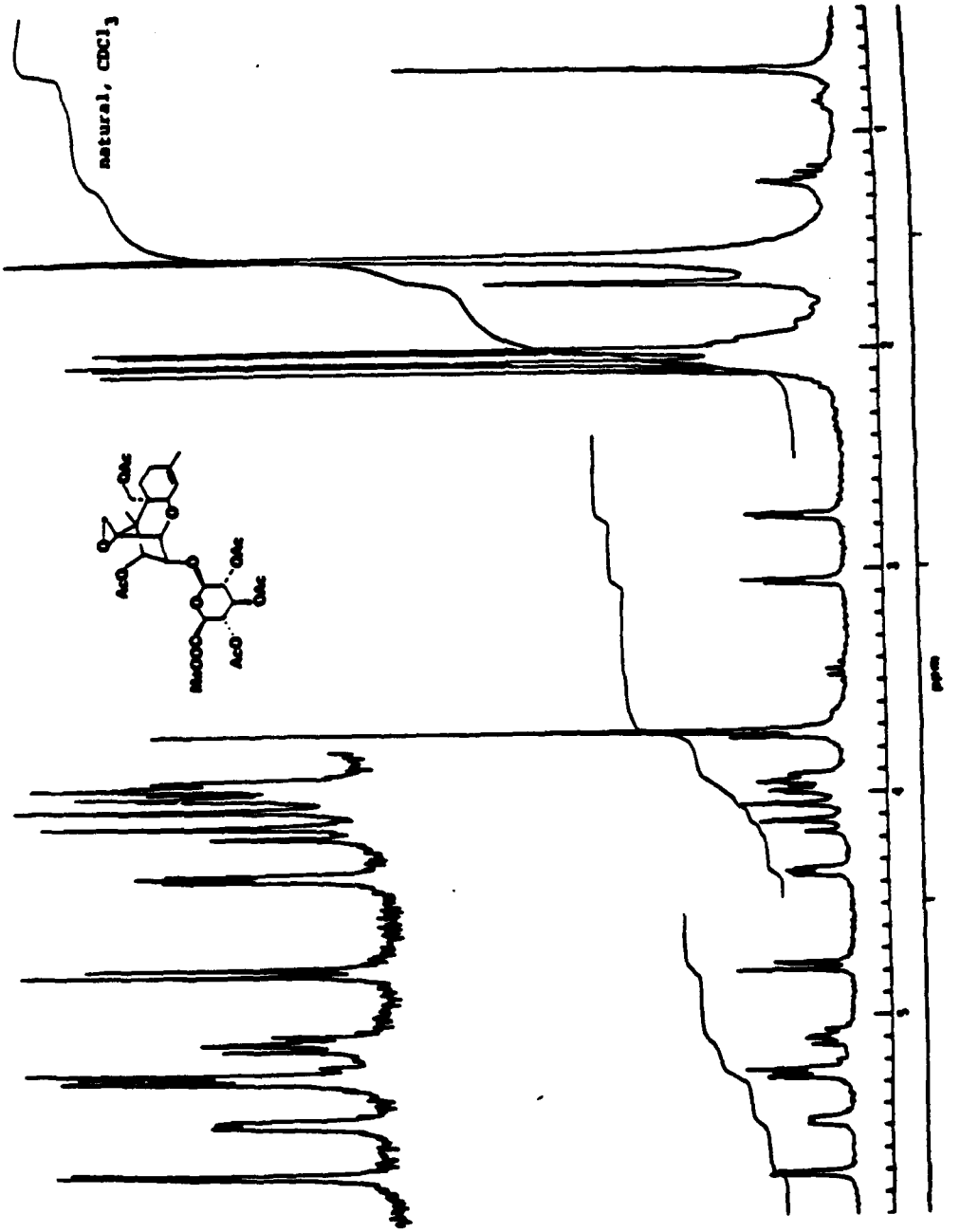
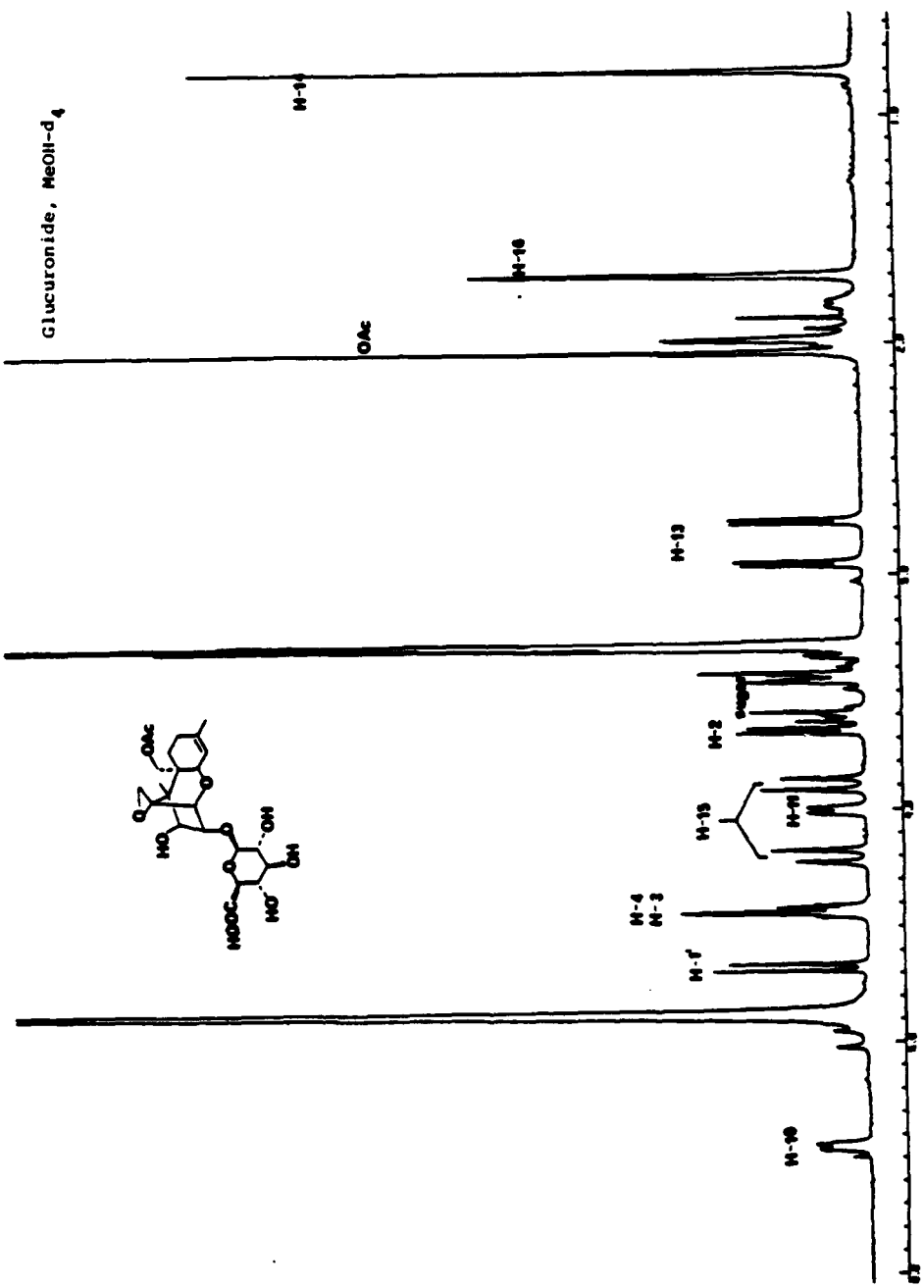
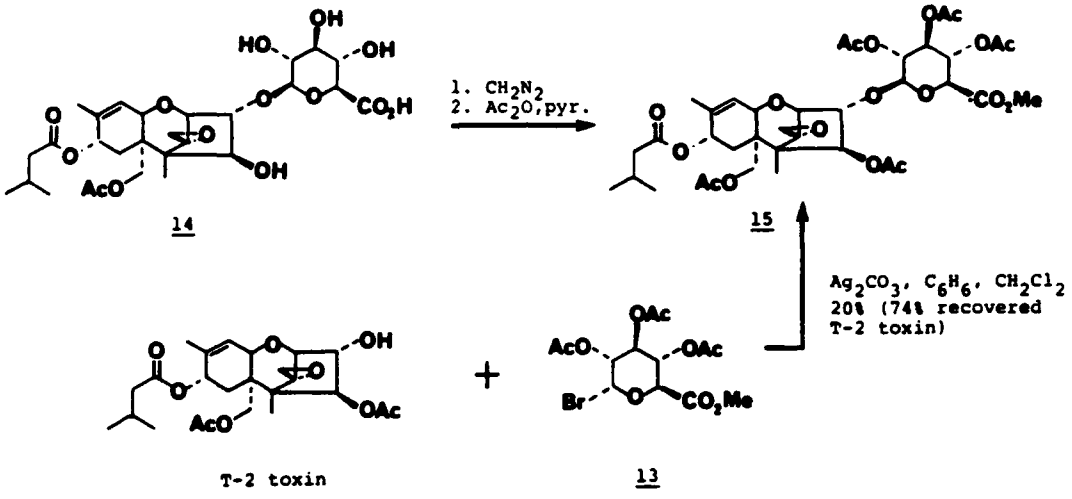


Figure 3 250 MHz ^{1}H NMR Spectrum of Glucuronide 11



treated approximately 1 mg of glucuronide 14 with diazomethane, and then with acetic anhydride in pyridine to afford the peracetate methyl ester 15. This compound was identical to the product of the Koenigs-Knorr reaction of T-2 toxin and bromosugar 13[9] (20% yield, 74% recovered T-2 toxin). The 250 MHz ^{1}H NMR spectra of synthetic and in vitro derived 15 are provided in Figures 4 and 5. These experiments unambiguously establish that the structure of this metabolite is correctly described by formula 14. The 250 NMHz ^{1}H NMR spectrum of 14 appears in Figure 6.



14 15

T-2 toxin 13

3. Studies on the Chemical Synthesis of Trichothecene Glucuronic Acid Conjugates

The determination of structure of in vivo generated metabolites is a formidable undertaking especially when only very small quantitites of metabolite can be purified. As noted previously, Marletta and Recchia have isolated two glucuronides from anguidine metabolism studies in vivo that so far have not been structurally characterized. We hope to solve this problem through chemical synthesis. This would also provide sufficient quantities of these compounds for biological characterization.

The syntheses of trichothecene-glucuronides will in many cases require the selective deprotection of the sugar blocking groups in the presence of acyl functionality which may be present in the trichothecene nucleus. For this reason, bromosugar 13, used previously in our syntheses of 12 and 15, is not suited for synthesis of the "real" glucuronide metabolites. Toward this goal, we have accomplished the preparation of bromosugar 19 and imidate 20. These compounds appear to be suited for use in Koenigs-Knorr glycosidation reactions.[10]

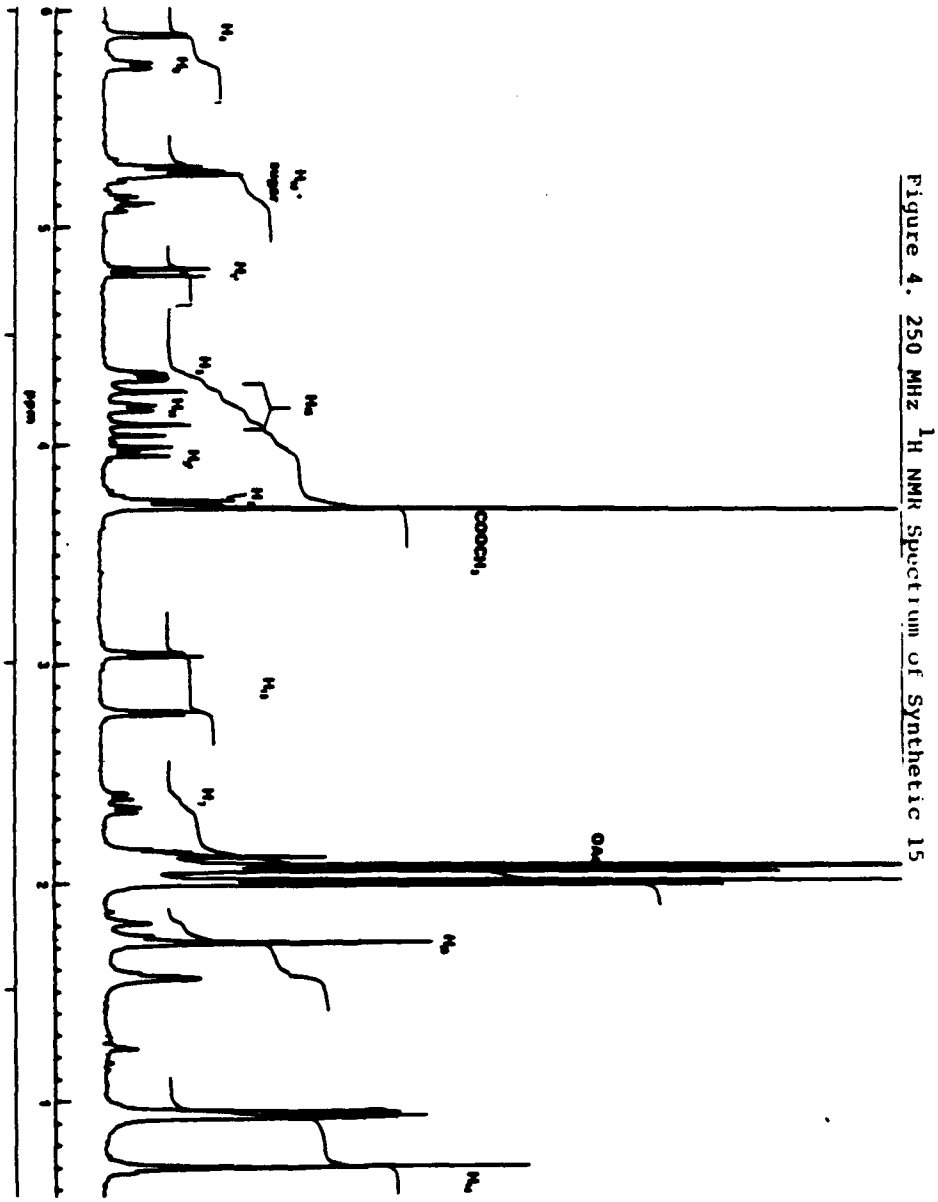


Figure 4. 250 MHz ^{1}H NMR Spectrum of Synthetic 15

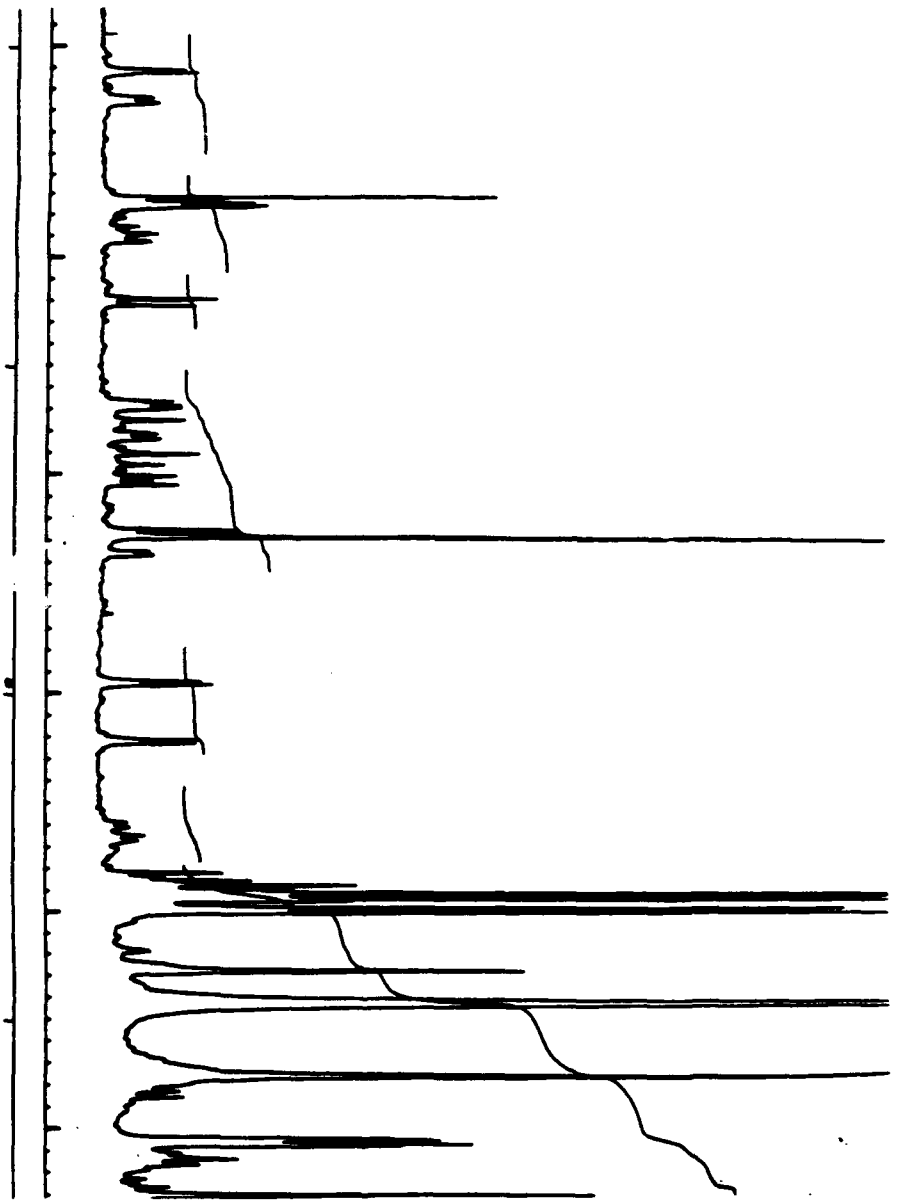


Figure 5. 250 MHz ^{1}H NMR Spectrum of In Vitro-Derived 15 in $CDCl_3$

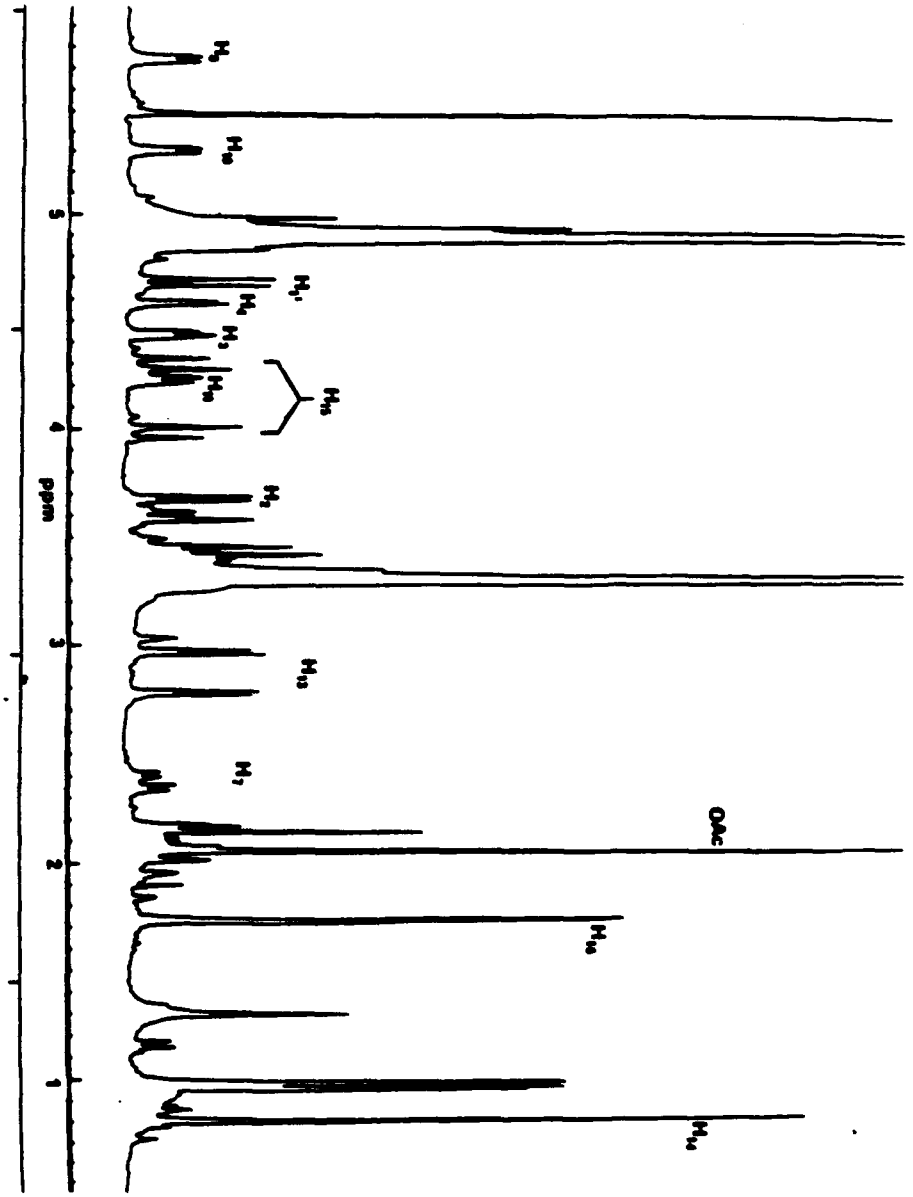
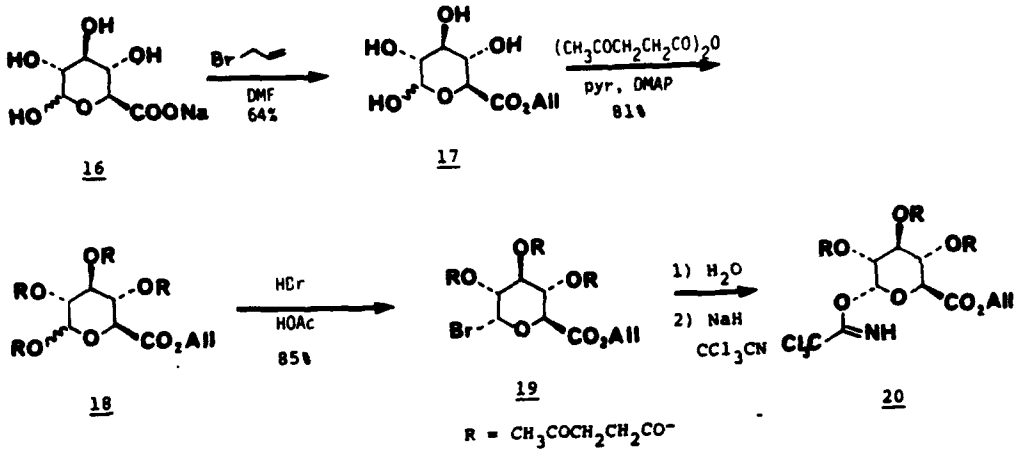


Figure 6. 250 MHz ^{1}H NMR Spectrum of Glucuronide 14 in CD_3OD



Moreover, the allyl and levulinate ester blocking groups can be cleaved under mild conditions in the presence of acetates.[11,12] Use of these reagents in the synthesis of *in vivo* and *in vitro* metabolites (e.g., 11, 14) will be studied in the next contract period.

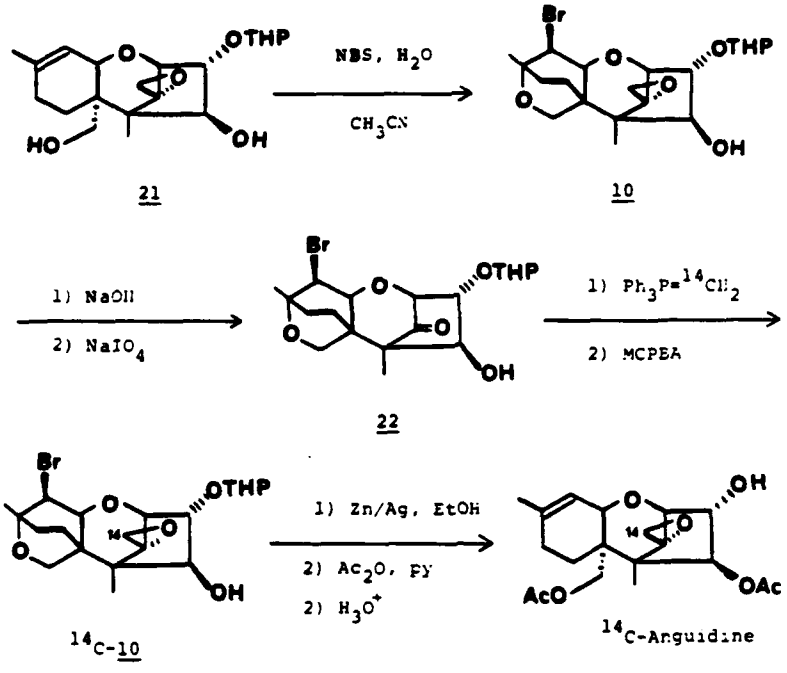
4. Progress Towards the Synthesis of ^{14}C-Anguidine

A potential problem with use of ^{3}H-labelled anguidine for *in vivo* and *in vitro* metabolism studies is that the tritium label can be lost via an oxidative process. Hence, some metabolites may escape detection when ^{3}H-labelled anguidine would ensure that all metabolites are detected. Our original plan for synthesis of ^{14}C-anguidine involved the degradation of anguidine to ketone 22, at which point the ^{14}C-label would be introduced by a Wittig reaction. The resynthesis of anguidine from 22 is directly analogous to the final stages of our verrucarol synthesis[13] (see also Scheme I for the synthesis of 4-acetoxyscirpendiol (8)).

The proposed degradation of 21 to 22, however, could not be accomplished in spite of considerable effort on our part. The epoxide functionality of 10 and 21 proved inert towards a wide variety of reagent, including KOH in DMSO or dioxane at 100°C, known to open

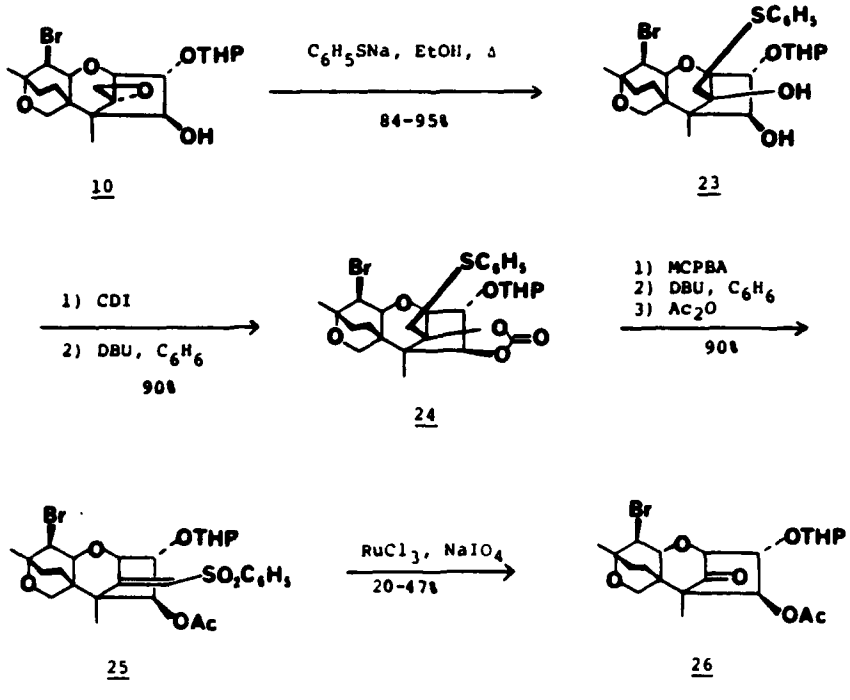
Scheme III

Proposed Synthesis of ^{14}C-Anguidine



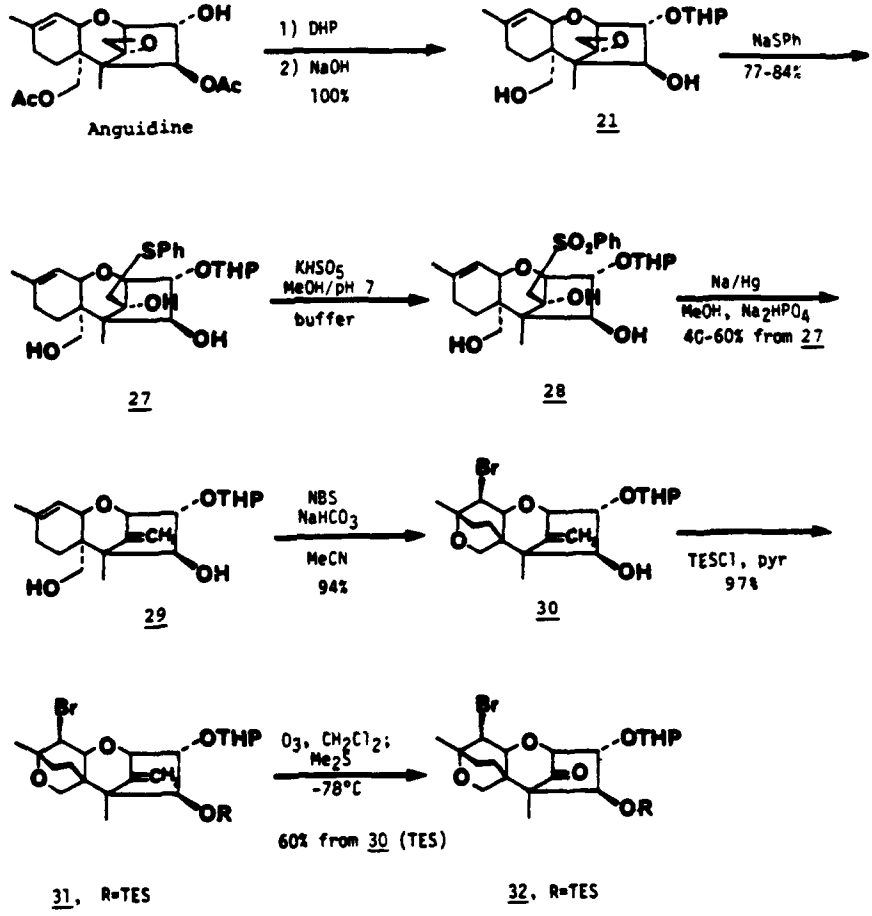
simple epoxides.[14] The reaction of 10 with a large excess of C_6H_5SNa (40-60 equiv.) in EtOH at reflux (20-26 h) was successful, and made possible the initial degradation outlined in Scheme IV.[15]

Scheme IV



(For preparation of 10, see Scheme I). The oxidative cleavage of 25 to 26, however, proceeded in less than acceptable yield so we developed a more efficient degradation as summarized in Scheme V.

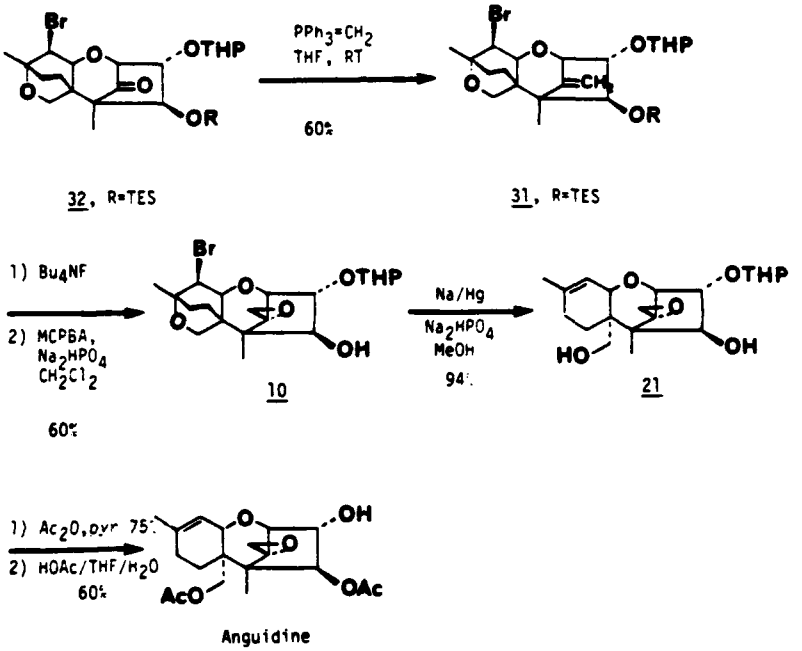
Scheme V



The key step of this new degradation is the sodium amalgam reduction of β-hydroxysulfone 28 which gives good yields of 29. After protection of the 9,10-olefin and C.4 hydroxyl group, ozonolysis of the 12,13-double bond in 31 afforded the desired ketone 32.

Methodology for resynthesis of anguidine from 32 is summarized in Scheme VI. Although several of the steps still must be optimized, the combination of Schemes V and VI define a variable route for synthesis of ^{14}C-anguidine. This work will be completed in the next contract period.

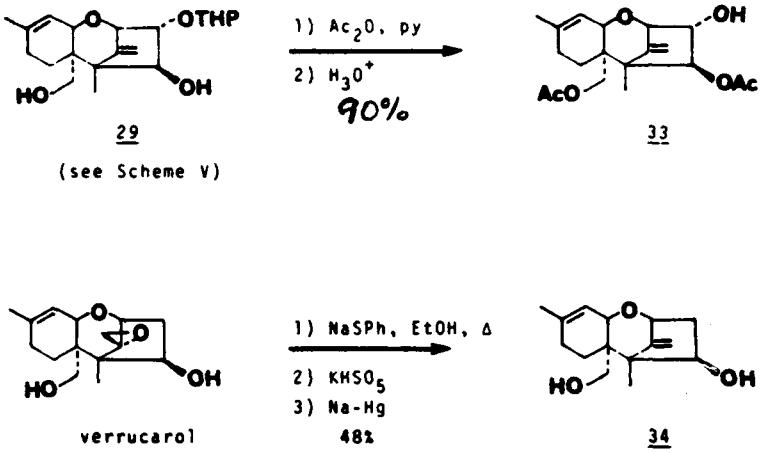
Scheme VI



5. Synthesis of 12,13-Deoxyanguidine and 12,13-Deoxyverrucarol

A characteristic structural feature of the trichothecene mycotoxins is the 12,13-epoxide group. Very recently two groups have reported the isolation of 12,13-deoxyvomitoxin from *in vivo* and *in vitro* metabolism studies of vomitoxin from *in vivo* and *in vitro* metabolism studies of vomitoxin (deoxynivalenol).[16] Since the 12,13-epoxide group appears to be associated with the adverse biological properties of these mycotoxins,[17] it is likely that this 12,13-deoxygenation reaction (performed by microorganisms in the gut) serves as a detoxification process. We suspected that a similar deoxygenation process might occur in *in vivo* studies of other trichothecenes, and decided to develop a methodology for synthesis of such compounds. This would provide authentic samples for identification of *in vivo* metabolites as well as provide reasonable quantities for biological characterization. Syntheses of 12,13-deoxyanguidine (33) and 12,13-deoxyverrucarol (34), therefore, are summarized in Scheme VII.

Scheme VII

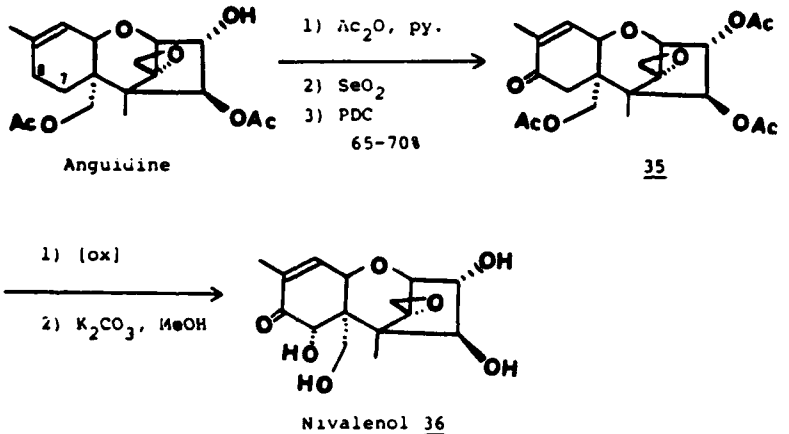


6. Progress Towards the Synthesis of Nivalenol

At the outset of this contract, the MIT group planned to study the metabolism of nivalenol as well as anguidine. Since a commercial source of nivalenol was not then available, we decided to synthesize this mycotoxin from anguidine. Our original synthetic plan is summarized in Scheme VIII. Procedures for effecting these conversions have been published, but the yields reported for the two oxidation steps (at C.8 and C.7, respectively) are very poor.[18] We prepared 35 from anguidine by a three step procedure. First, triacetoxyscirpenol was treated with

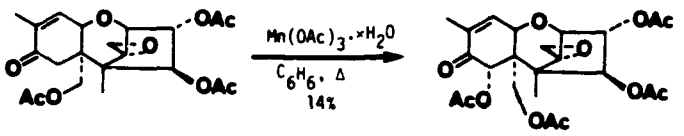
Scheme VIII

Proposed Synthesis of Nivalenol from Anguidine



5 equiv. of SeO_2 in a mixture of HOAc and Ac_2O[19] at 110°C for 40 min. This reaction afforded the 8β-alcohol which was oxidized using pyridinium dichromate (PDC)[20] to give 35 in 65-70% overall yield.

Grove has reported that 35 can be hydroxylated at C.7 by using $Pb(OAc)_4$.[18b] We, however, were unable to reproduce this result and consequently examined a number of alternatives including hydroxy(tosyloxy)iodosobenzene[21] which gave no reaction. Other procedures involving the oxidation of enolates[22,23] derived from 35 were unsuccessful for reasons which we originally attributed to the incompatibility of acetate protecting groups to these strongly basic reaction conditions. A small scale oxidation of enone 35 with $Mn(OAc)_3 \cdot xH_2O$[24] afforded acetylated nivalenol 37 in 14% yield. The stereochemistry of this compound was determined by correlation of ^1H NMR spectral data with that

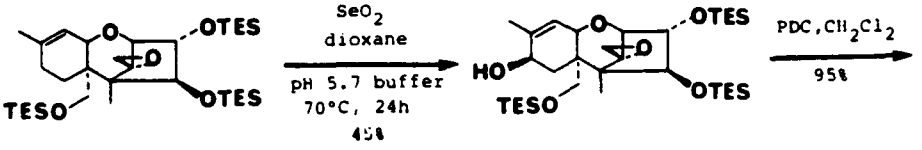


35 37

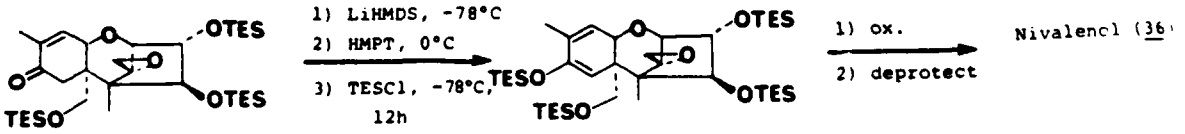
of authentic 37 reported by Grove.[18b] This step, however, is still too low yielding to be useful on a preparative scale.

Given the difficulties with enolate oxidations noted above, we decided to use triethylsilyl (TES) protected ketone 40 as a key intermediate (see Scheme IX). The starting material, 38, was prepared

Scheme IX

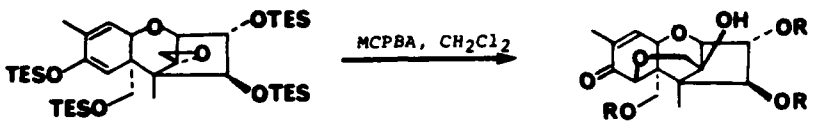


38 39



40 41

in 90% yield by treatment of scirpentriol with Et_3SiCl and pyridine.[25] Optimal conditions for allylic oxidation of 38 involved treatment with 0.5 equiv. of SeO_2 at 70°C in dioxane buffered to pH 5.7 with 50 equiv. of aqueous KH_2PO_4-K_2HPO_4. This reaction afforded 39 in 45% yield along with 40% of recovered 38 and 5% of the C.16-alcohol. Oxidation of 39 with PDC[20] afforded 40 in very high yield. Numerous attempts to oxidize the enolate of 40 to 41 could be accomplished in 60-65% yield under carefully controlled conditions. Attempts to convert 41 to nivalenol, however, also proved to be difficult. A result that suggests that further modifications of the synthesis may be required is the isolation of a compound identified as 42 from a MCPBA oxidation of 41.[27]



41 42 (R=TES)

Additional work on nivalenol has been postponed pending completion of the DAS and T-2 metabolism studies being performed in collaboration with Dr. Marletta. If nivalenol work is resumed, nivalenol will be purchased from Romer Labs.

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Segment 3 - Toxicology

P.M. Newberne

A.E. Rogers

Segment 3 - Toxicology
P.M. Newberne and A.E. Rogers

The aims of this segment have been to characterize the injury to biological systems by DAS (anguidine), in a comparative manner, using mice, rats, and more recently guinea pigs. The acute toxicity has been determined in rats and mice by oral and intraperitoneal exposure and, in conjunction with Professor Wogan's group, preliminary effects by topical administration have been identified. Most of our work during the contract years was done using mice. This was because of the known effects of trichothecenes on the hematologic and immune systems and the relatively greater ease of examining these systems in mice. While numerous studies have been done, only a description of a typical study and the observations will be given here.

Young mice of the CD-1 strain, obtained from Charles River Breeding Laboratories, Wilmington, MA were housed in isolation animal facilities. They were dosed or injected with the compound DAS, dissolved in 10% DMSO, and examined at times ranging from one hour to two weeks after exposure. We determined the response from exposure by aerosol in a single study and found an LC_{50} to be about 11 mg/kg body wt. Following the single inhalation study, we examined responses to single doses of DAS given by gastric intubation (i.g.) or by intraperitoneal injections. While the LD_{50} varied among various groups of test mice, the i.g. route was about 20 mg/kg body weight and the i.p. route about 15 mg/kg. Following the determination of the LD_{50} we settled on a sublethal dose of 8 to 10 mg/kg body weight for use in examining a number of parameters. Some of the results are provided here and others can be found in the various quarterly reports.

Hematological Findings

After exposure to DAS hemoglobin concentrations and erythrocyte counts were slightly elevated between 5 and 12 hours inclusive. By 48 hours, they were both slightly decreased. Reticulocyte counts were high at 9 and 12 hours, but were also elevated in vehicle-treated controls at 12 hours. Total leukocyte counts were elevated within 1 hour, peaked at 2-3 hours after administration of toxin, rapidly declined until they reached subnormal levels after 18 hours with a nadir at 2-3 days. Elevations were due to an initial rise in numbers of circulating lymphocytes by 1 hour, and a subsequent elevation in neutrophil counts by 4 hours. Lymphocyte counts remained elevated until 6 hours, after which they declined and reached subnormal levels by 18 hours. Neutrophil counts remained elevated from 3 to 12 hours, and then declined to subnormal levels by 24 hours after treatment with the toxin. After seven days, quantitatively, all values were comparable to control figures. Table 5 lists sequential changes to 96 hours post exposure.

Clinical Observations

Table 5 . Hematological Values Following DAS, 10 mg/kg Body Weight

Hours post-treatment	Hemoglobin g/dl	Erythrocytes $x10^6/\mu l$	Reticulocytes %	Leukocytes $x10^3/\mu l$	Neutrophils $x10^3/\mu l$	Lymphocytes $x10^3/\mu l$
1	14.6±0.5[a]	7.8±0.3	4.6±3.0	9.5±0.5	1.1±0.3	8.1±0.7
2	13.6±0.3	7.7±0.2	8.8±2.9	9.2±1.3	1.8±0.4	7.4±1.0
3	13.4±0.4	7.7±0.2	7.1±1.9	10.9±2.6	2.6±1.0	8.3±1.7
3(control)	13.7±0.3	7.5±0.2	10.9±2.2	3.8±1.3	0.2±0.1	3.3±1.3
4	14.5±0.5	8.2±0.3	15.5±2.3	17.2±4.2	5.1±1.9	12.0±3.0
5	15.3±0.2	8.2±0.2	7.3±0.8	13.2±2.1	2.9±0.6	10.1±1.7
6	15.3±0.4	8.3±0.3	6.0±1.8	6.8±1.6	1.7±0.6	5.0±1.3
6(control)	14.7±0.2	7.8±0.1	12.6±1.4	4.5±0.6	0.6±0.1	3.9±0.5
9	15.4±0.7	7.9±0.4	19.9±4.3	5.9±1.1	3.3±0.4	2.6±1.6
9(control)	12.4±0.5	7.3±0.2	15.8±1.2	5.1±0.8	0.8±0.2	4.2±0.8
12	16.2±0.5	9.0±0.4	25.5±7.0	7.2±1.3	3.7±0.7	2.8±0.5
12(control-vehicle	13.3±0.4	6.9±0.2	19.4±4.6	6.0±0.9	1.2±0.2	4.9±0.6
18	13.5±0.3	7.6±0.2	9.3±1.7	3.1±0.3	1.7±0.2	1.6±0.1
24	13.4±0.9	7.9±0.6	9.3±0.9	2.7±0.2	0.9±0.2	1.8±0.3
48	12.6±0.4	6.8±0.2	3.1±1.3	2.2±0.3	0.4±0.1	1.7±0.3
96	12.4±0.7	6.7±0.3	7.2±2.1	2.8±0.3	3.7±0.7	2.5±0.3

[a]Values are means (±SEM) for separate groups of 5 or 6 mice.

Aside from paleness, there were no significant signs or symptoms in mice exposed to DAS. They might appear normal at one observation point and in two hours they would be dead. The acuteness of demise without clinical evidence of illness in mice is apparently in contrast to larger animals such as swine.

Observations at Necropsy

After 6 and 9 hours, the small intestine was usually dilated and filled with fluid. Similar changes were also found in mice killed after 12 and 18 hours, by which time colonic contents were also fluid. Intestinal contents were blood-tinged throughout the lower intestinal tract in those mice that died. At 48 hours, the spleen appeared small, but by 96 hours, splenomegaly was evident in two mice. The thymus was consistently small in all treated mice at both 48 and 96 hours.

Histopathology

Mice treated with DAS developed mitotic arrest, quickly followed by karyorrhexis in germinal regions of the intestinal mucosa, lymph nodes, thymic cortex, splenic white and red pulp, and in the bone marrow. Karyorrhexis was also observed in the intraepithelial and subepithelial populations of lymphocytes of the small intestinal villi, particularly in the Peyer's patches. Soon afterwards, mitotic activity resumed in the damaged tissues and cell debris was rapidly cleared. Compensatory hyperplasia was often observed in previously damaged tissues 3-4 days following exposure. Rates of onset of karyorrhexis and recovery were variable among the different tissues and the severity of injury was variable among different mice. In any single mouse, the degree of injury to the intestinal mucosa varied with level to level and, in some individuals, from crypt to crypt.

Intestinal epithelium: Mitotic activity in the duodenum, jejunum and ileum ceased completely during the first 2 hours after treatment. Within 2 hours, epithelial cells in the crypts lacked clear cellular detail and some contained small, eosinophilic, intracytoplasmic particles. The numbers of these particles increased markedly until 12 hours, and then decreased. Intracellular inclusions were generally round, multiple, and located in cells of the lower half of the crypts. Some bodies contained basophilic debris which was Feulgen positive indicating probable nuclear debris. In severely affected crypts, large numbers of round bodies were present in an aggregate that became extruded into the lumen of the crypt. Neutrophils appeared in the lamina propria by 2-3 hours and some emigrated into the lumen of damaged crypts after 6 hours. Mitotic activity returned in some mice after 5 or 6 hours in the duodenum and after 9 hours in the ileum. At 24 hours, there were only minimal amounts of debris and some crypts were atrophic. Mitotic rates were highest at 48 and 96 hours, compared to control mice. Some crypts were distorted and hyperplastic and in villi were shortened.

Intestinal lamina propria: Numerous pyknotic and karyorrhectic nuclei were found after 1 hour in the lamina propria of the villi and, to a lesser extent, between the crypts. Pyknotic cells which appeared to be intraepithelial lymphocytes were found in the villus epithelium and fragments of karyorrhectic nuclei were present in clusters after 2 hours. In mice with severe crypt lesions, the lamina propria was edematous and there were increased numbers of neutrophils in capillaries near damaged crypts. Pyknotic and karyorrhectic lymphoid nuclei were numerous in and around foci of intestinal lymphoid tissue and Peyer's patches. Karyorrhexis, and subsequent depletion, was also observed in germinal centres of Peyer's patches between 1 and 12 hours after treatment.

Thymus: Mitotic activity was absent in both the cortex and medulla within 2 hours after exposure to DAS. Karyorrhectic nuclei were present in the cortex during the first 6 hours; by 12 to 24 hours the cortex was largely destroyed. Macrophages cleared out the cellular fragments and most debris disappeared after 24 hours. After 48 and 96 hours, the cortex contained for the most part, only epithelial and reticular cells. Mitotic activity in the thymic medulla and cortex had resumed in some mice by 96 hours.

Splenic white pulp: Mitotic lymphoblasts were consistently seen in controls but, within 1 hour following treatment, karyorrhexis was present in follicles. After 12 hours, mitotic activity had resumed and the cell debris was largely removed.

Lymph nodes: Mitotic activity was absent in germinal centres between 1 and 12 hours inclusive, but then resumed. Varying degrees of karyorrhexis were found in germinal centres of active follicles at 1 hour after treatment and between 2 and 12 hours, pyknotic and karyorrhectic nuclei were infrequently observed in the paracortical and medullary regions of the nodes. Macrophages contained lymphoid cellular debris between 2 and 4 hours following exposure to DAS.

Splenic red pulp: Within 3 hours after exposure, small fragments of karyorrhectic nuclei were observed in hematopoietic populations, and numbers of immature hematopoietic precursors declined during this period. Regenerative activity was evident after 48 hours, first in the megakaryocytes population and later in myeloid cells.

Table 6 lists some typical mitotic counts from five high power fields at different levels of the gut and in the thymus, spleen, and bone marrow. Injury scores of the same mice are also included.

Potential Protective Factors Against DAS Toxicity

Having established the qualitative and to some extent, the quantitative effects and the time course of injury from DAS in

Table 6 . Mitotic Counts and Injury Scores in Mice Exposed to 10 mg/kg DAS

		Hours Following Treatment								
Duodenum		0	1	2	3	6	12	24	48	96
[1] Mitoses	1)	19	20	0	1	21	2	40	76	70
	2)	50	9	0	4	2	14	31	39	60
[2] Injury Score	1)	0	0	1	0	1	3	1	0	0
	2)	0	0	0	1	1	4	1	0	0
Ileum	1)	18	12	0	2	4	1	25	65	57
Mitoses	2)	40	44	0	0	2	4	18	30	45
Injury Score	1)	0	1	0	2	1	2	1	0	0
	2)	0	0	0	1	2	4	2	0	0
Thymus	1)	10	17	2	8	0	0	0	0	3
Mitoses	2)	14	18	4	2	1	0	0	0	11
Injury	1)	0	0	0	1	0	4	4	0	0
	2)	0	0	1	1	1	4	4	1	0
Spleen	1)	22	5	0	0	0	0	6	7	10
Mitoses	2)	14	2	0	1	0	0	4	3	7
Injury	1)	0	2	2	3	3	2	0	0	0
	2)	1	2	3	3	1	1	0	0	0
Bone Marrow	1)	0	0	1	0	1	2	3	3	0
Injury	2)	0	0	0	1	0	2	3	2	0

[1] Mitoses, number in 5 high power fields, except 1 HP Field in thymic cortex.

[2] Injury ranging from none = 0 to 4 = destruction most susceptible cells. These are representative means of counts and injury scores from 40 mice.

mice, we have examined numerous candidates for prevention or alleviation of the toxic effects. Using WR2721 as a reference agent we have used a variety of compounds synthesized by Professor Buchi's laboratory, only one of which provided any degree of protection. It was already known that the one compound, L-2-oxothiazolidine, which was effective in our hands, promotes the intracellular synthesis of glutathione and in this manner offers protection from some toxic agents.

The progress in identifying protective agents synthesized for specific proposed protective actions was so slow that this segment was dropped at the end of the contract covered by this report.

We have tested additional materials including bentonite, charcoal, intestinal sterilants, antibiotics, among others, with disappointing results. Charcoal seemed to offer some protection but this was variable from one trial to another.

Following these rather extensive investigations we turned to other natural and synthetic compounds which are readily available. These include vitamin C, vitamin E, difluoromethylornithine, B-carotene and some of the retinoids, levamisole, lithium carbonate and DMSO. Data from these studies will be presented in the next progress report.

Recovery of Hematological and Immunological Systems After Exposure To DAS

We have further examined the recovery of the hematopoietic and the immune systems over time up to 42 days, following a single sublethal dose of DAS. Male mice of the CD-1 strain were injected with DAS, 10 mg/kg body weight. Some mice were sacrificed at time points noted in tables 7 through 12. There were reductions in leukocytes, thrombocytes, hemoglobin and hematocrit during the first seven days post-exposure, observations made earlier. In addition there was depression of a proliferative response of splenic lymphocytes to concanavalin A (Con A), phytohemagglutinin A (PHA), pokeweed mitogen (PWM), and lipopolysaccharide (LPS) during the first two weeks following exposure. The recovery time was about three weeks. The response to mitogens appeared to indicate that the first subset of lymphocytes affected is the mature lymphocytes, followed by other subsets without any particular preference. The blast forms in the thymus, spleen, lymph nodes and gut wall are particularly susceptible. Injury from DAS to the immune system is according to a dose response curve. A fascinating aspect of the injury is that it is an *in vivo* phenomenon; in culture the DAS toxicity to freshly isolated lymphocytes appears to be low. This suggests an *in vivo* mediated effect of the toxin.

Proliferative Capacity of the Hematopoietic Stem Cells of the Bone Marrow in Mice Pretreated with DAS

able 7: Effect of one single dose 10 mg/kg BW DAS IP in CD-1 male mice on white blood cell, platelet, hemoglobin, hematocrit at 1,3,7,14,21,28 and 42 days post-treatment.

ime fter reatment	Animals per Group	WBC/ml x10^6	Platelet/ml x10^8	Hemoglobin g/dl	Hematocrit %
ntreated	20	8.2±0.6	6.8±0.6	16.8±0.6	49.7±0.6
d	5	6.4±0.4	2.3±0.2^e	16.0±0.2^c	41.8±0.4^e
d	5	3.1±0.1^e	2.1±0.02^e	14.5±0.4^e	35.8±1.4^e
d	8	7.7±1.3	7.5±0.7	14.4±0.3^e	45.5±1.1^d
4d	8	6.9±0.9	8.2±1.1	15.8±0.2^d	48.0±0.7
1d	8	7.6±0.8	10.6±1.1^d	17.2±0.3	49.8±0.4
8d	8	10.6±1.1^b	6.9±0.8	17.3±0.3	50.3±0.9
2d	8	10.5±1.3^a	7.1±0.6	17.6±0.3	50.0±0.7

ean ± SEM
= $p < 0.05$ b = $p < 0.025$ c = $p < 0.01$ d = $p < 0.005$ e = $p < 0.0005$

able 8. Body weight, spleen weight, spleen cells in CD-1 male mice pretreated one dose of 10 mg/kg BW DAS intraperitoneal at 7,14,21,28,42d

ime fter reatment	Body Weight g	Spleen Weight g	Total Spleen cell/ g spleen x10^6	No. Spleen cell/ ml x10^6
ntreated	32.9±0.5	0.13±0.01	933.8±46.9	41.4±2.7
d	29.7±0.5^d	0.25±0.01^e	652.9±31.2^d	57.0±6.1^c
4d	32.7±0.7	0.18±0.01^e	576.3±44.3^e	35.4±4.8
1d	33.1±0.3	0.16±0.01^e	788.8±40.7^a	43.2±2.9
8d	34.3±0.9	0.18±0.01^e	1015.4±45.9	59.1±4.7^d
2d	36.3±1.2^d	0.15±0.01^b	865.1±44.2	43.0±2.2

lean ± SEM, 8 animals per group except untreated 20 mice.
a = $p < 0.05$; b = $p < 0.025$; c = $p < 0.01$; d = $p < 0.005$; e = $p < 0.0005$.

ble 9. Response of lymphocytes from spleens of control and DAS treated mice to conA* at 1,2,3 and 4 weeks post treatment

me	^{3}HTdR uptake (cpm)** DAS	Control	Stimulation Index*** DAS	Control
wk	93209±15502 (5)	128136±21956 (4)	93.1±18.8	138.6±31.6
	157840±23595 (8)	176989±26066 (3)	414.6±74.2	623.6±163.5
	218075±25556 (5)	264487±30790 (4)	525.9±104.8	395.6±137.3
	147223±24422 (6)	149075±57273 (3)	541.6±86.1	701.9±301.8
	154977±30248 (8)[a]	265121±19489 (4)	567.4±92.5	857.6±110.8

ean ± SEM, ( ) = No. of animals, DAS 10 mg/kg BW IP
ConA final concentration 5 ug/ml
= $p < 0.05$
Differential uptake (stimulated uptake - unstimulated uptake)
*SI $\frac{\text{(stimulated uptake)}}{\text{(unstimulated uptake)}}$

Table 10. Response of lymphocytes from spleens of control and DAS treated mice to PHA* at 1,2,3 and 4 weeks post treatment

Time	^{3}HTdR uptake (cpm)** DAS		^{3}HTdR uptake (cpm)** Control		Stimulation Index*** DAS	Stimulation Index*** Control
1 wk	7014±1100[a]	(5)	25865±9235	(4)	8.1±1.4[c]	24.5± 3.9
2	11477±1673	(8)	17518±8813	(3)	28.5±2.3[b]	51.9±16.3
3	10317±1711	(5)	12757±3917	(4)	28.3±8.9	28.6± 6.5
4	5905±825	(6)	6404±541	(3)	21.9±3.7	30.5± 4.5
6	10146±1681	(8)	10472±2080	(4)	36.4±6.6	36.9±9.4

Mean ± SEM , ( ) = No. of animals, DAS 10 mg/kg BW IP.
*PHA final concentration 10 ug/ml
a = $p < 0.05$; b = $p < 0.025$; c = $p < 0.005$.
**Differential uptake (stimulated uptake - unstimualted uptake)
***SI $\frac{\text{(stimulated uptake)}}{\text{(unstimulated uptake)}}$

Table 11. Response of lymphocytes from spleens of control and DAS treated mice to PWM* at 1,2,3 and 4 weeks post treatment

Time	^{3}HTdR uptake (cpm) DAS		^{3}HTdR uptake (cpm) Control		Stimulation Index DAS	Stimulation Index Control
1 wk	15425±3233	(5)	32822±3979	(4)	15.9±3.6[b]	36.1±6.9
2	4001±510	(8)	4167±316	(3)	11.0±1.1[a]	15.3±1.9
3	2721±656	(5)	3181±893	(4)	7.6±1.6	9.2±2.8
4	1970±189	(6)	1993±641	(3)	7.7±1.1	9.8±3.7
6	1805±267	(8)	3062±561	(4)	6.4±1.0[a]	10.8±2.6

Mean ± SEM, ( ) = No. of animals, DAS 10 mg/kg BW IP.
*PWM final concentration 3.125 ug/ml
a = $p < 0.05$; b = $p < 0.025$
**Differential uptake (stimulated uptake - unstimulated uptake)
***SI $\frac{\text{(stimulated uptake)}}{\text{(unstimulated uptake)}}$

Table 12. Response of lymphocytes from spleens of control and DAS treated mice to LPS* at 1,2,3 and 4 weeks post treatment

	^{3}HTdR uptake (cpm)**		Stimulation Index***	
Time	DAS	Control	DAS	Control
1 wk	22666±2950 (5)	30781±9627 (4)	22.9±3.1	35.3±15.5
2	9315±1443 (8)	12685±3132 (3)	24.4±3.9[a]	41.6±3.9
3	6880±708 (5)	8752±1951 (4)	12.0±3.5	20.9±5.7
4	5737±324 (6)	7058±2228 (3)	21.8±2.6	31.9±12.8
6	7670±863 (8)	9540±1257 (4)	26.6±2.9	31.8±3.6

Mean ± SEM, ( ) = No. of animals, DAS 10 mg/kg BW IP.
*LPS final concentration 25 ug/ml
a = $p < 0.025$
**Differential uptake (stimulated uptake - unstimulated uptake)
***SI $\frac{\text{(stimulated uptake)}}{\text{(unstimulated uptake)}}$

The profound effect of DAS on the hematopoietic system, particularly the bone marrow. When trichothecenes are given by oral intravenous or intraperitoneal application, the tissues with the most severe alterations are those with a component of rapidly dividing cells such as the gut, lymphoid and hematopoietic tissue, and testes. Gross examinations reveal that the bone marrow of DAS treated mice is pale, edematous and may contain focal hemorrhages. In chickens given a single dose of DAS, necrosis of the erythroid and granulocytic regions of the bone marrow was observed at 6 hours and was most severe at 24 hours. Necrosis was accompanied by marked cellular depletion.

Bone marrow contains only a small fraction of stem cells and those that are present do not have distinctive morphology, biochemistry or antigenicity. Ludkowiez et al., 1963, suggested that the small marrow lymphocyte may be a pluripotent hemopoietic stem cell but the means for identification is imprecise. Murine spleen colony assay is the only means for functional determination currently available which can characterize the proliferative capacity and/or the self renewal of the hemopoeitic stem cell and thus provides a valuable tool for studying toxic effects on bone marrow and other components of the hemopoietic system. The aim of the present study was to investigate the effect of DAS on the proliferative capacity of hemopoietic stem cells in treated animals at various time points via different routes of administration.

Male weanling mice of the CD-1 strain were housed four per cage in climate-controlled facilities with SaniChip bedding and free access to feed (chow) and water and a 12 hour light-dark cycle. Male Fischer rats, approximately 100 grams in body weight were also purchased from Charles River Breeding Laboratories, housed the same as the mice except that they were caged individually.

Diacetoxyscirpenol (DAS) or anguidine was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10% in distilled water. DAS was given in a single dose of 10 mg/kg BW intraperitoneal (IP) or 6.5 to 25 mg/kg BW topical on skin to a mouse or rat as indicated in each experiment.

Blood samples were obtained via retroorbital venous plexus for total and differential white blood cell counts, platelet counts, hemoglobin and hematocrit determinations. Bone marrow cells were aseptically collected by dissecting both femurs free of attached tissue, cutting the femur at the epiphysis and flushing the shaft with 1 ml of RPMI 1640. Single cell suspensions were prepared by successive passage of the marrow cells through 22 gauge needles. Nucleated cells were enumerated in the hemocytometer. Viability of the cell suspensions was determined by using the trypan blue exclusion method.

Bone marrow stem cells thus obtained were examined by the

spleen colony forming method (CFU-S) originally described by Till and McCulloch (1961).

Isolated bone marrow cells of 1 to 5 x 10^4 cell/0.5 ml from DAS exposed and untreated control mice were injected intravenously into the lateral tail vein of 35 $\pm$ 2 day old male CD-1 mice previously irradiated with 850 Rad of ^{137}Cs. Recipients were killed 8-10 days following cell transfer. Spleens were removed, weighed and fixed for 24 hours in Bouin's fixative. Colonies on the spleen were enumerated with a magnifier and histological sections were made of random colonies to confirm their morphology. Results were expressed as colonies per spleen or colony forming unit (CFU) per femur.

$$\text{CFU/Femur} = \frac{(\text{cell/femur})\ (\text{colony/spleen})}{\text{no. cell injected}}$$

$$\text{Fraction CFU remaining} = \frac{\text{Test CFU/Femur}}{\text{Control CFU/Femur}}$$

Histopathology was by routine methods. At sacrifice organs were removed and placed in 10% buffered formalin pH 7.4 except that bone was placed in Zenker solution. Tissue sections were prepared and processed by standard procedures for light microscopy. All data were analyzed by analysis of variance, student t-test and linear regression.

RESULTS

Hematology: Effect of a single dose of 10 mg/kg BW DAS on white blood cells, platelets, hemoglobin and hematocrit in CD-1 male mice at 4,8,24,72 and 168 hours were shown in Table 13. There was a transient leukocytosis at 4 and 8 hours after DAS exposure which gradually developed into leukopenia on the third day following treatment; three values returned toward pretreatment level 4 days later or seven days post exposure. The total platelet count was decreased by DAS exposure with a nadir at 3 days post treatment. There were slight increases in hemoglobin and hematocrit at the early time points. There were no significant differences in the volume of packed red blood cells in mice treated with DAS at any time point up to 72 hours, but at this point the hematocrit was decreased 15% from the untreated control.

The differential white blood cell counts were calculated as absolute number of cells per cubic millimeter. Results are shown in Table 14. One day after exposure the percentage of neutrophils increased from 36 to 70% but returned to control levels by seven days. During the first four to eight hours following DAS administration, there was an increase in the immature leukocytes in the peripheral blood but this generally returned to near normal values by seven days.

Table 13. Effect of one single dose of 10 mg/kg BW DAS, ip on white blood cell, platelets,hemoglobin and hematocrit in CD-1 male mice at 4, 8 hr 1, 3 and 7 days.

Time after Treatment	WBC/ml x10^6	Platelet/ml x10^8	Hemoglobin g/dl	Hematocrit %
0 hr	7.8±0.5	7.4±1.7	15.0±0.5	41.8±1.1
4 hr	37.9± 5.0^d	4.3±0.6	17.2±0.3^b	45.2±0.9^a
8 hr	30.5±3.6^d	3.1±0.5^a	16.8±0.4^a	42.8±1.2
1 d	6.4±0.4	2.3±0.2^b	16.0±0.2	41.8±0.4
3 d	3.1±0.1^d	2.1±0.02^b	14.5±0.4	35.8±1.4^b
7 d	8.2±1.9	7.2±0.6	14.7±0.4	47.0±1.3^c

Mean ± SEM, 5 animals per group.

a = $p < 0.05$, b = $p < 0.025$, c = $p < 0.01$, d = $p < 0.005$.

Table 14 Absolute number of cells per cumm in CD-1 male mice treated with DAS 10 mg/kg BW IP at 4, 8 hr and 1, 3 and 7 days

Time after treatment	WBC/ cumm	Lymphocyte	Neutrophil	Eosinophil	Basophil	Monocyt.	Ringform	Immatur
0 hr	7750±480	4702±427(60.6)	2822±300(36.6)	71±53(0.6)	31±18(0.5)	105±26(1.4)	19±18(0.2)	-
4 hr	37900±5010^{d}	26232±3492^{d}(69.2)	8929±1300^{d}(23.6)	322±142(1.4)	-	316±81^{a}(1.0)	1863±639^{b}(4.4)	94±93 (0.4)
8 hr	30500±3580^{d}	21858±3333(70.6)	8455±811^{d}(28.8)	-	-	-	187±80(0.6)	-
1 d	6400±429	2618±839^{b}(40.4)	3766±272(59.4)	-	-	-	-	16±15 (0.2)
3 d	3150±127^{d}	2929±125^{d}(92.2)	208±39(7.5)	-	-	13±7^{c}(0.33)	-	-
7 d	8200±1925	4810±998(60.8)	2871±827(33.6)	60±36(0.6)	-	139±69(2)	320±157(3)	-

Absolute no. of cells per cumm = $\frac{\%\ \text{differential}}{100} \times$ WBC/cumm , ( ) = % differential

Mean ± SEM, 5 animals per group.
a = $p < 0.05$, b = $p < 0.025$, c = $p < 0.01$, d = $p < 0.005$.

The number of bone marrow cells began to decline at 4 hours; this continued to a nadir by 24 hours and subsequently recovered to the untreated control level by the seventh day. There was no change in bone marrow cell counts of DMSO treated animals, as shown in Table 15.

The endogenous colony forming capacity was obtained from an experiment in which colonies in the spleen of mice were counted 9 days after irradiation with ^{137}Cs, 600 to 1000 Rads. The number of endogenous colonies observed is related to the radiation dose. 600 Rads was too low and 1000 Rads was too high. 850 Rads was the dose of choice; this amount of radiation was required to suppress the appearance of endogenous colonies but still permits an average of less than one endogenous colony per spleen.

The relationship between the number of normal nucleated marrow cells and the number of colonies formed in the spleen of CD-1 male mice is shown in Figure 1. There was a linear relationship between the number of cells injected and the mean number of colonies per spleen $r = 0.93$, $y = 6.08 + 0.000183x$

y = colony per spleen

x = number of cell injected

No colonies were found in non-irradiated mice.

Spleen weight of DAS treated animals was significantly increased at 7 days post treatment. In irradiated mice, spleen weight varied according to the number of cells injected; the weight increased as the number of cells injected was increased.

Figure 2 illustrates the effect of DAS on colony forming units at various time points. Bone marrow cell suspensions were from donor mice treated with one single dose of either 8 or 10 mg/kg BW of DAS administered by intraperitoneal injection. The effect was more severe in mice treated with 10 mg/kg BW DAS compared to those given 8 mg/kg BW. CFU/Femur of both groups reached the nadir at 8 hours after treatment.

Figure 3 illustrates CFU in mice injected with nucleated marrow cells from donor mice pretreated i.p. with one dose of DAS 10 mg/kg BW, 10% DMSO, or untreated control, at 4, 8, 24 and 168 hours. Stem cells exhibited the greatest depression at 8 and 24 hours post treatment with DAS. DMSO treated animals had a slightly better response in colony forming units, an apparent mitogenic affect. Alternatively, DMSO may have had some influence on the colony stimulating factor which in turn enhanced the colony forming units.

The effect of DAS depended on time, dose and route of administration. These data will be presented later when the experiments are completed.

The present experiments clearly demonstrated that DAS in a single dose as low as 10 mg/kg BW, intraperitoneally to mice,

Table 15. Effect of DAS and DMSO on bone marrow cellularity of CD-1 male mice at 4, 8 hr and 1, 3, 7 and 9 days

	Bone Marrow Cellularity x 10^6 per ml	
Time	DAS	DMSO
0 hr	17.4±1.9(7)	17.4±1.9(7)
4hr	10.5±1.0[a](4)	19.5±2.6(2)
8hr	7.0±0.8[b](8)	17.8±1.3(3)
1d	3.5±1.0[b](6)	21.4±3.0(3)
3d	6.9±1.9[b](4)	18.5±0.0(2)
7d	18.7±0.1(2)	22.7±1.6(4)
9d	22.4±1.1(4)	ND

Mean ± SEM, ( ) = No. of animal, DAS 10 mg/kg BW IP

a = $p < 0.025$ ND = not determined

b = $p < 0.0005$

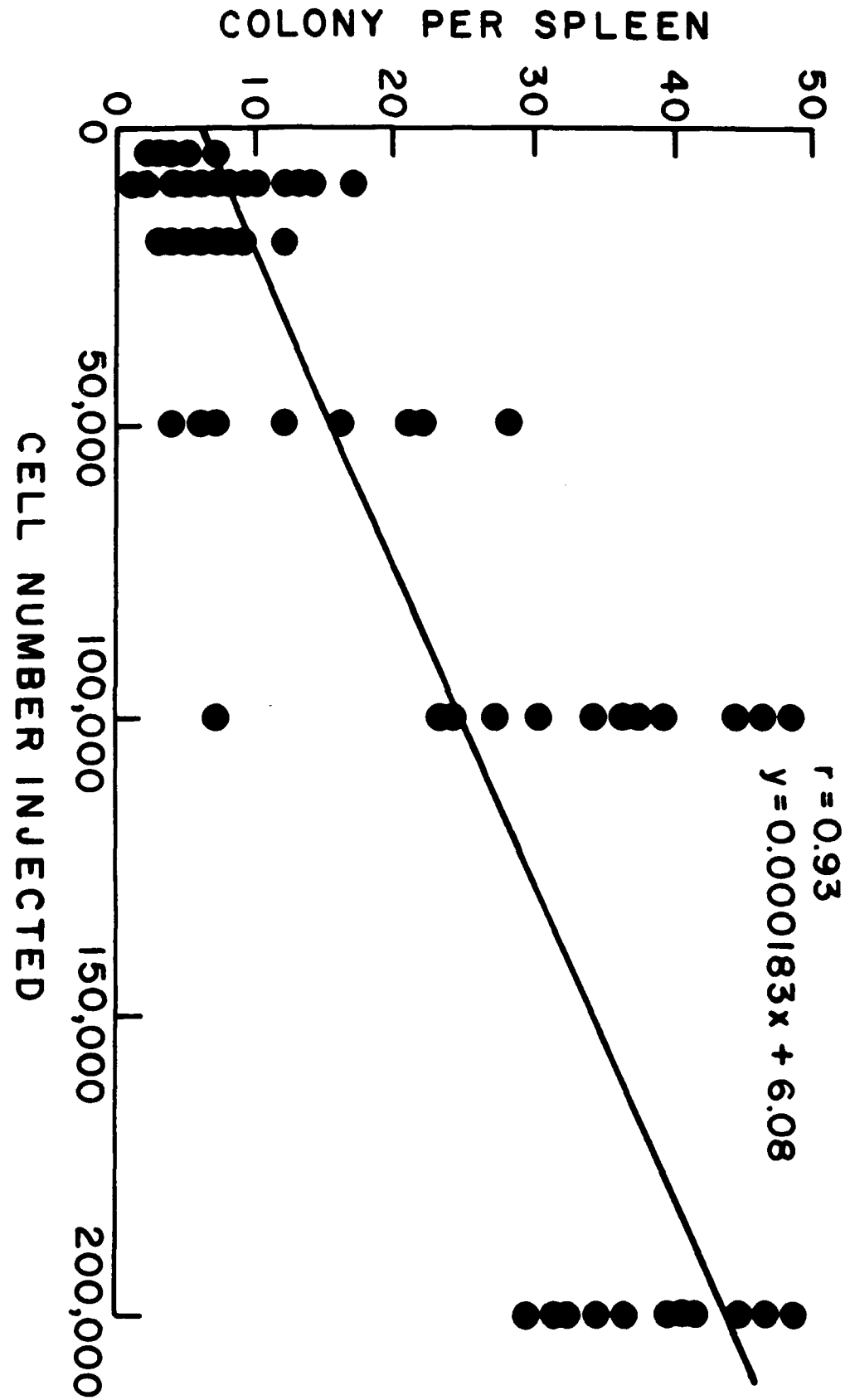

Figure 1. Relation of normal nucleated marrow cells isolated from the bone marrow and the number of colonies formed in irradiated mouse spleens.

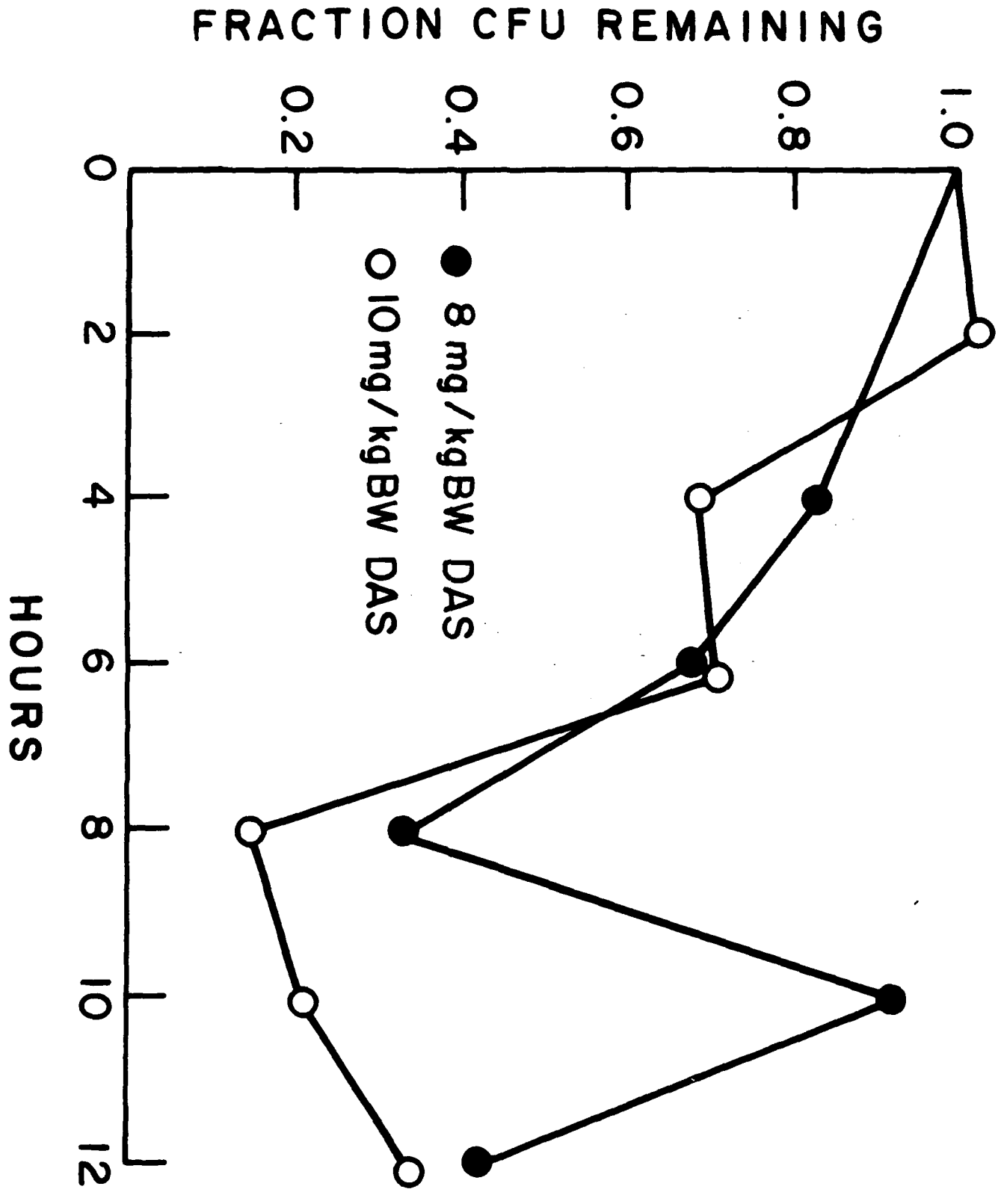

Figure 2. Effect of 8 or 10 mg DAS. Nadir in both groups was reached after 8 hours.

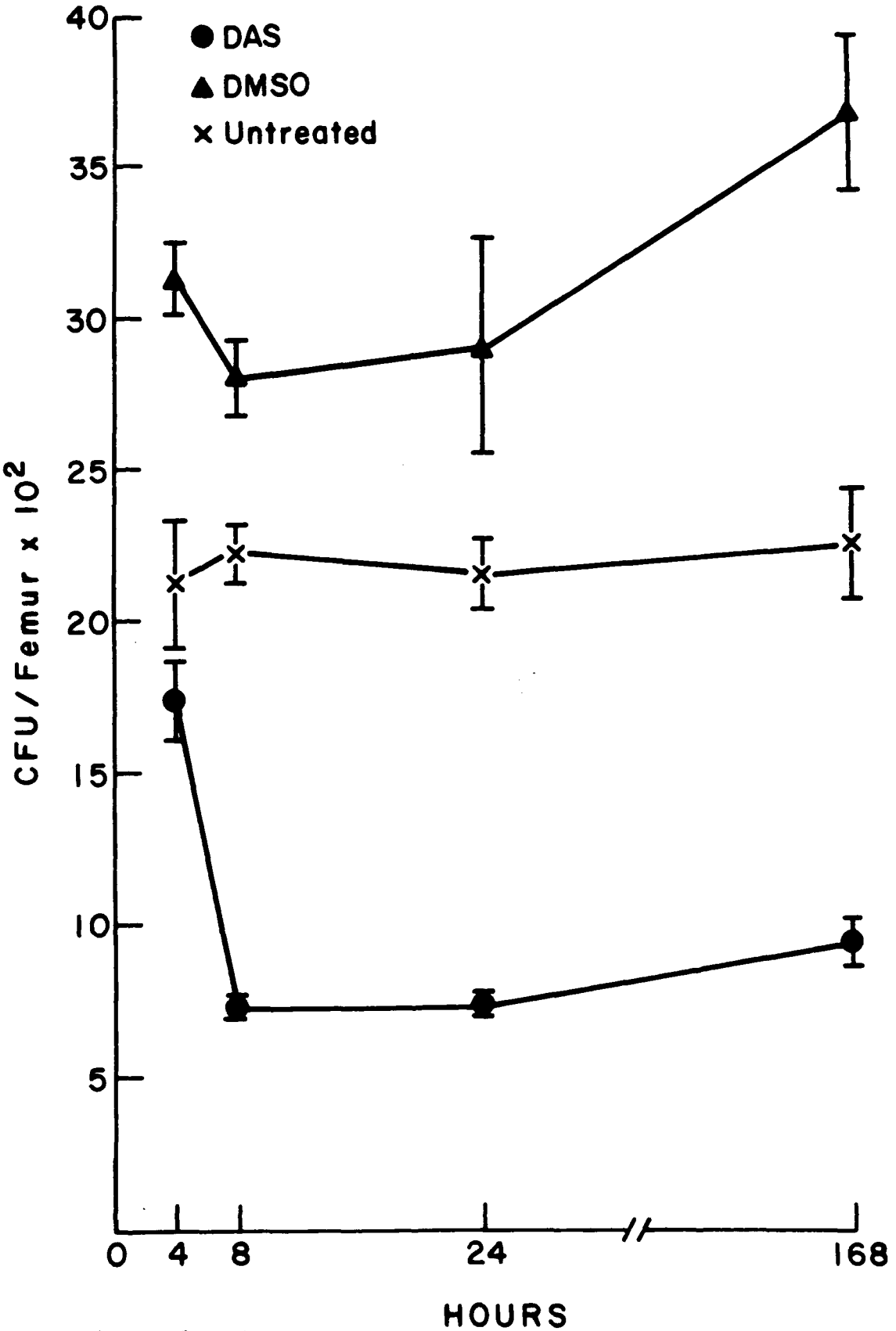

Figure 3. CFU in CD-1 male mice injected with femoral marrow cell from donor mice pretreated one dose of DAS 10 mg/kg BW, 10% DMSO or untreated control IP at 4, 8, 24 and 168 hours. Animals were 6-29 per group.

suppressed the bone marrow, both in terms of cell numbers and function. It took seven days for the number of cells to achieve the untreated level whereas the proliferative capacity of these cells reached 90% of control in 12 days.

The mode of action of DAS is thought to be irreversible blockage of protein synthesis, rather than DNA or RNA synthesis. Bone marrow is composed of many immature cells which renders them sensitive to DAS. Several factors, including cell to cell interaction, microenvironmental factors and humoral factors are to be considered as potential differences on the regulation of function of these bone marrow cells.

As noted earlier, we have continued to evaluate the testes as a means for sensitive detection of injury from DAS and to use this indicator in studying protective agents. Figures 4-12 illustrate some of our findings to date.

DISCUSSION

The distribution of lesions observed in tissues of mice exposed to a single sublethal dose of DAS, and other trichothecenes resembles that seen after exposure to ionizing radiation or to high doses of various anti-neoplastic chemicals, including alkylating agents, methotrexate, hydroxyurea, cytosine arabinoside, or colchicine. Thus, the acute lesions caused by DAS toxin are non-specific. However, because most other common mycotoxins do not cause such radiomimetic lesions, this pattern of injury could be used as a means of tentatively distinguishing trichothecenes from other mycotoxins.

Similar distributions of lesions have been described in various experimental animals given single doses of various trichothecene mycotoxins, namely nivalenol in mice; fusarenon-X in mice; neosolaniol in mice; fusarenon-X in rats, guinea pigs, cats and ducklings; and T-2 toxin in mice, cats, guinea pigs, and swine. In most of these reports, the pathogenesis of the lesions was not determined; microscopic changes were usually described in moribund or dying animals that had been given lethal doses of mycotoxin. Saito and Okubo (1970) reported sequential changes in mice at 6, 12 and 24 hours after doses of fusarenon, fusarenon-X, nivalenol and crude *Fusarium* extracts, but these investigators did not describe the lesions in detail. Thus, the observations in our studies provide a description of the early histological and sequential changes in acute sublethal DAS toxicosis.

The rapid onset within one hour of lympholysis in the intestinal epithelium, intestinal lamina propria, the Peyer's patches, and the germinal-centres of follicles illustrated that several populations of lymphocytes are exquisitely sensitive to this toxin at dose levels well below the median lethal dose. Thymic cortical lymphocytes were also extremely sensitive, but this population was not destroyed until 9 hours after exposure.

EFFECT OF ANGUIDINE ON TESTIS WEIGHT
testis wt g
1621
1215
1409
1603
days after treatment
1
3
7
30
60
90
cmpd
ANG
DMSO

FIGURE 4

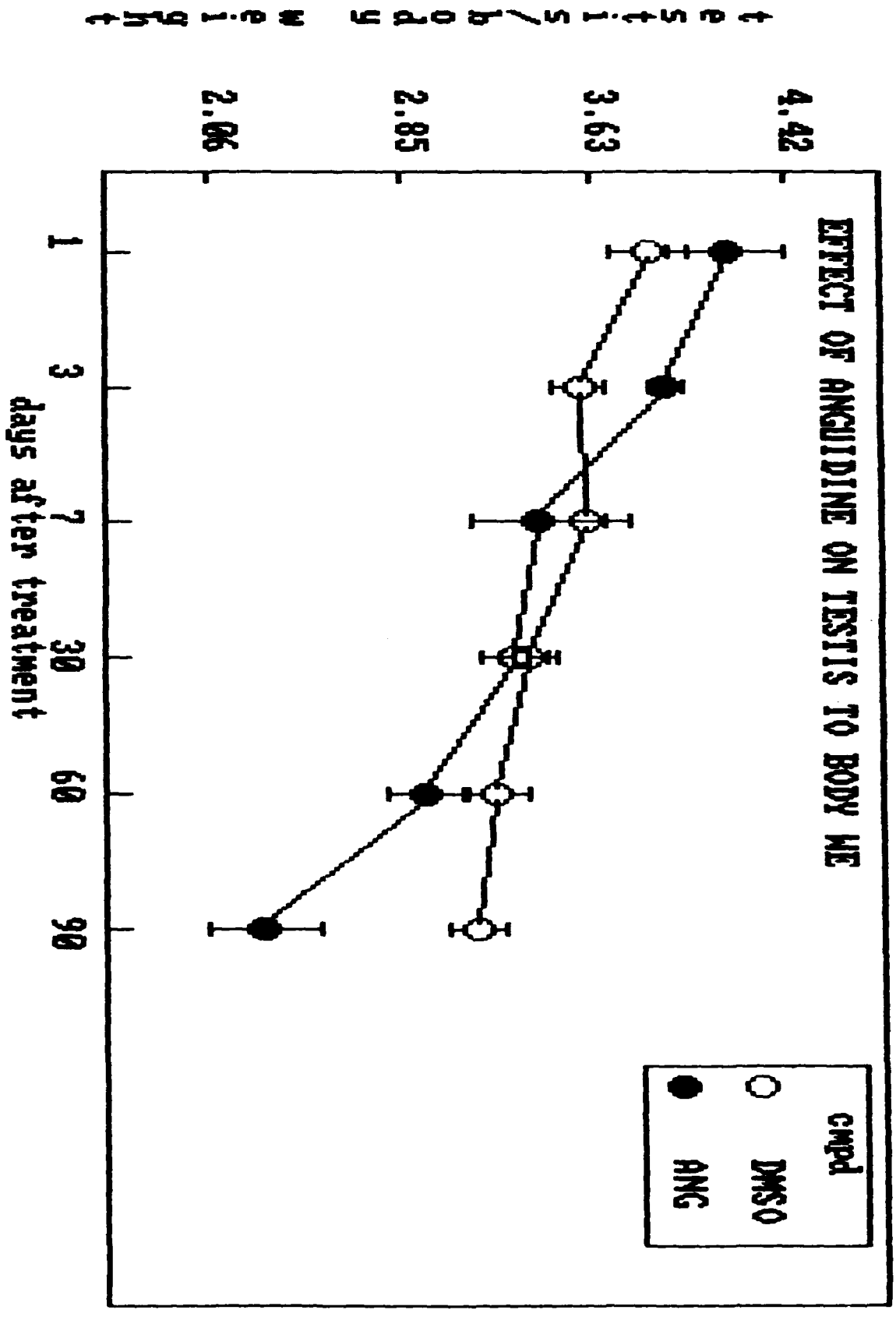
EFFECT OF ANGUIDINE ON TESTIS TO BODY WE
testis wt/body wt
4.42
3.63
2.85
2.06
1
3
7
30
60
90
days after treatment
cmpd
DMSO
ANG

FIGURE 5

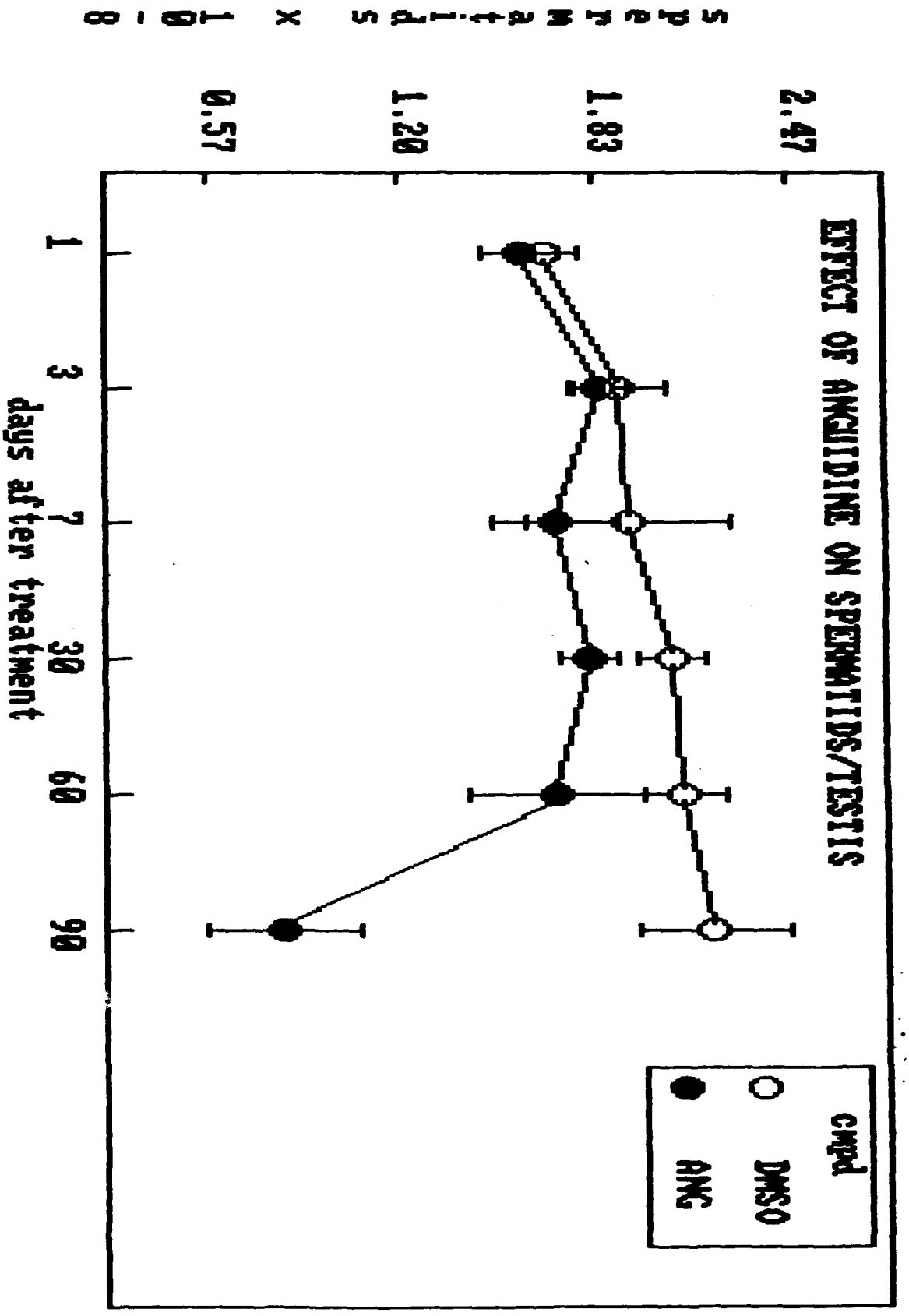
EFFECT OF AMGUIDINE ON SPERMATIDS/TESTIS
Spermatids x 10 8
2.47
1.83
1.20
0.57
1
3
7
30
60
90
days after treatment
cmpd
DMSO
AMG

FIGURE 6

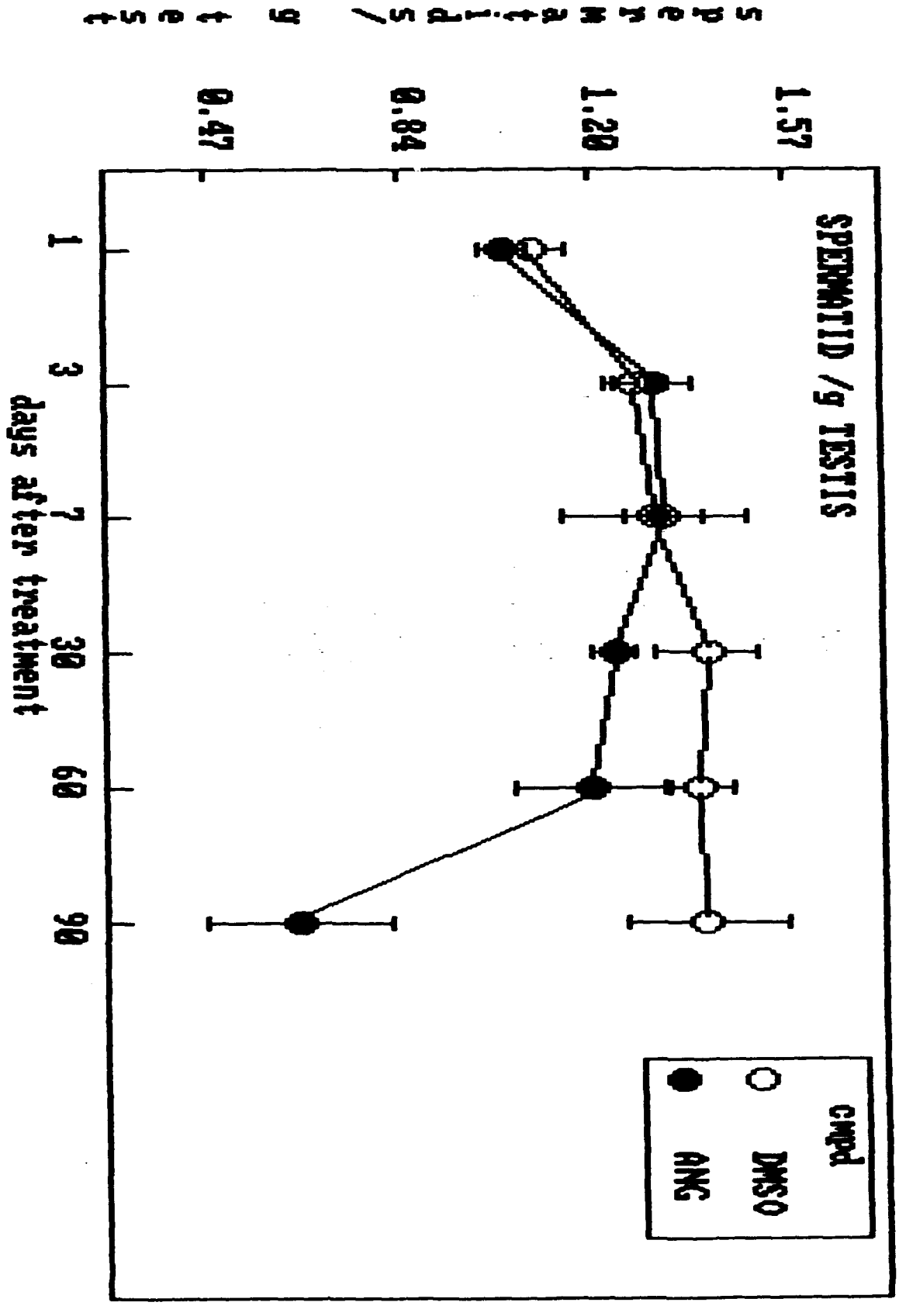
SPERMATID / g TESTIS
1.57
1.20
0.84
0.47
days after treatment
1
3
7
30
60
90
Cmpd
DMSO
ANC

FIGURE 7

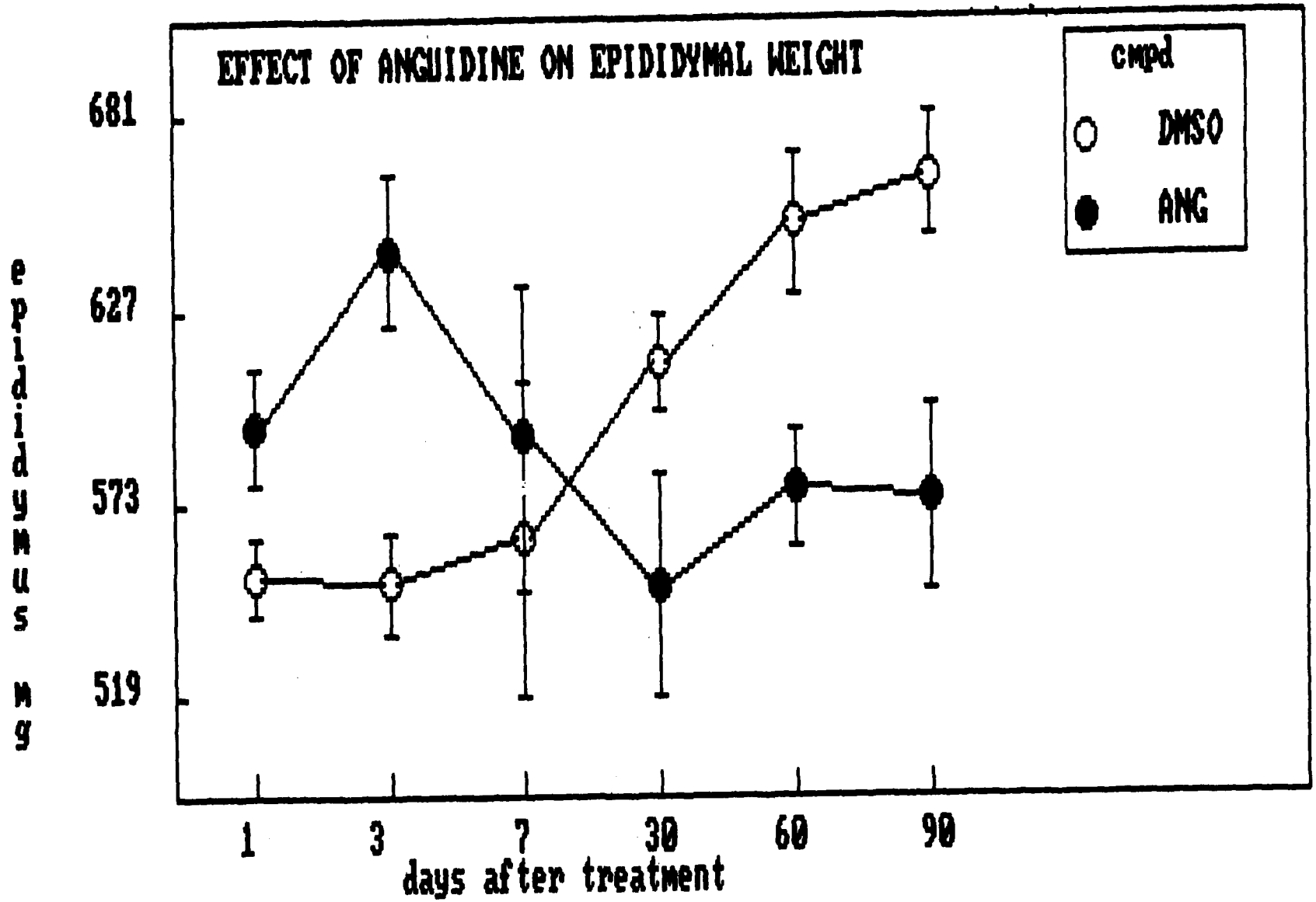
EFFECT OF ANGUIDINE ON EPIDIDYMAL WEIGHT
cmpd
DMSO
ANG
epididymus mg
681
627
573
519
1
3
7
30
60
90
days after treatment

FIGURE 8

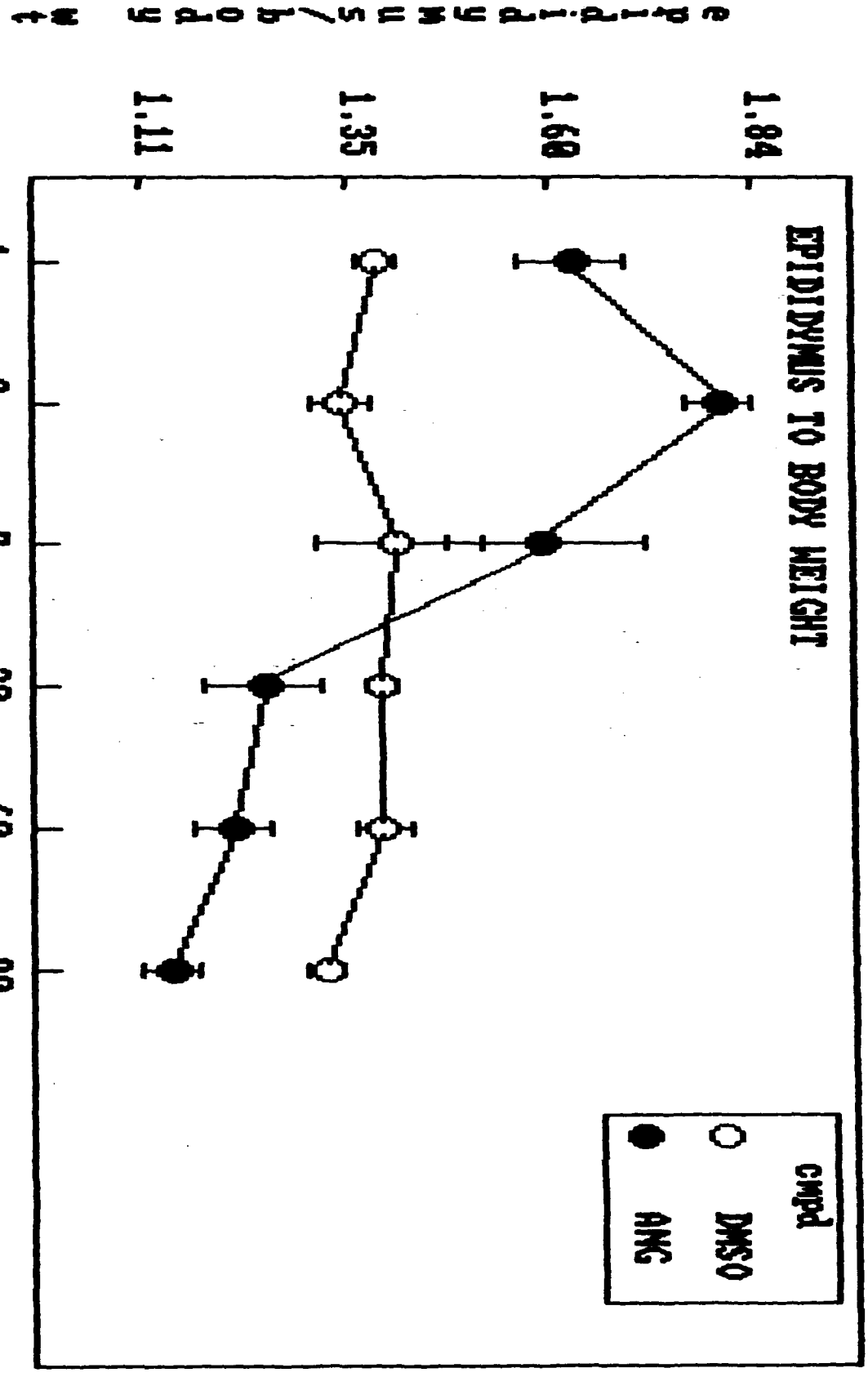
EPIDIDYMIS TO BODY WEIGHT
epididymis/body weight
1.84
1.60
1.35
1.11
1
3
7
30
60
90
days after exposure
Cmpd
DMSO
AMG

FIGURE 9

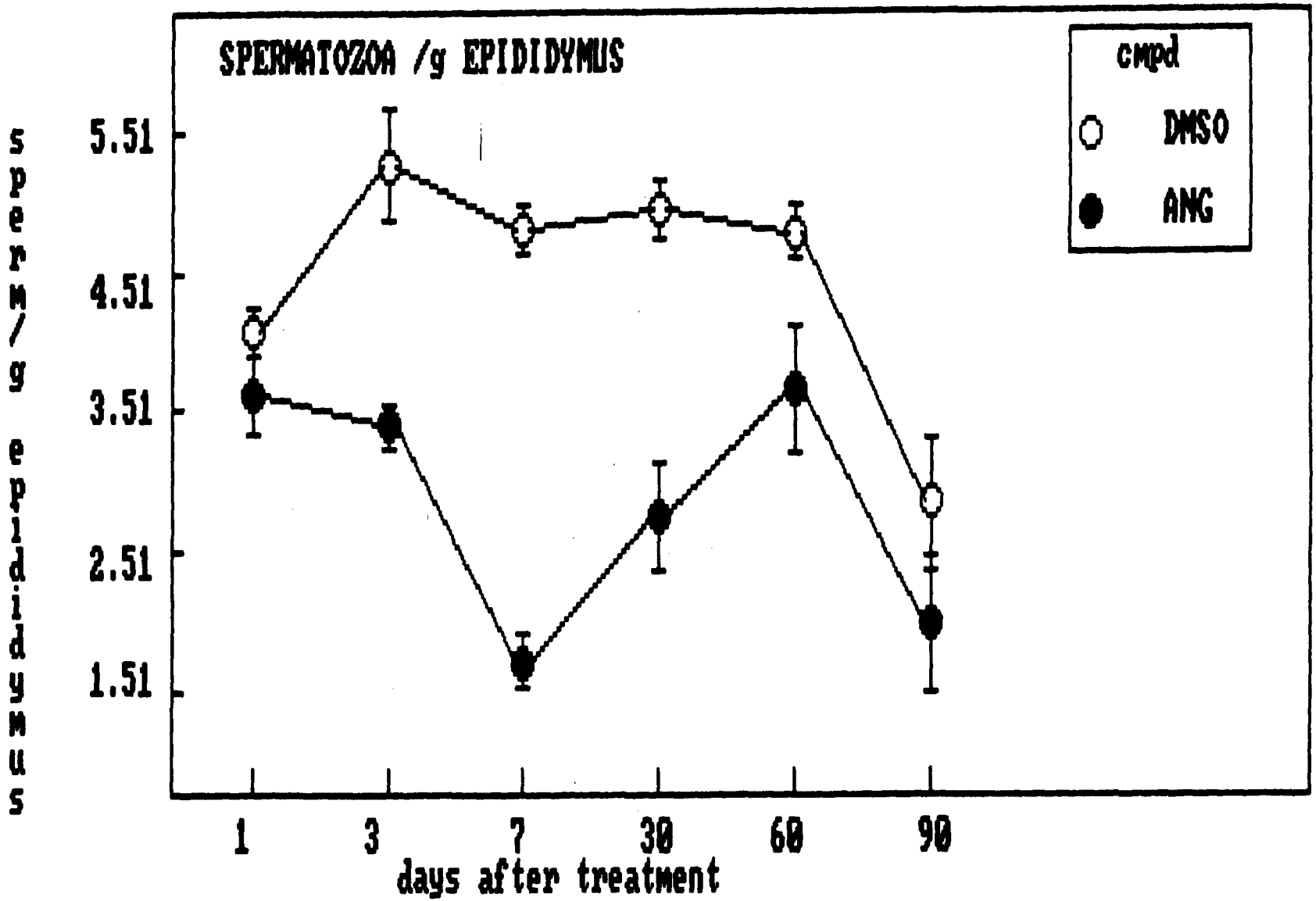
SPERMATOZOA /g EPIDIDYMUS
cmpd
DMSO
ANG
sperm/g epididymus
5.51
4.51
3.51
2.51
1.51
1
3
7
30
60
90
days after treatment

FIGURE 10

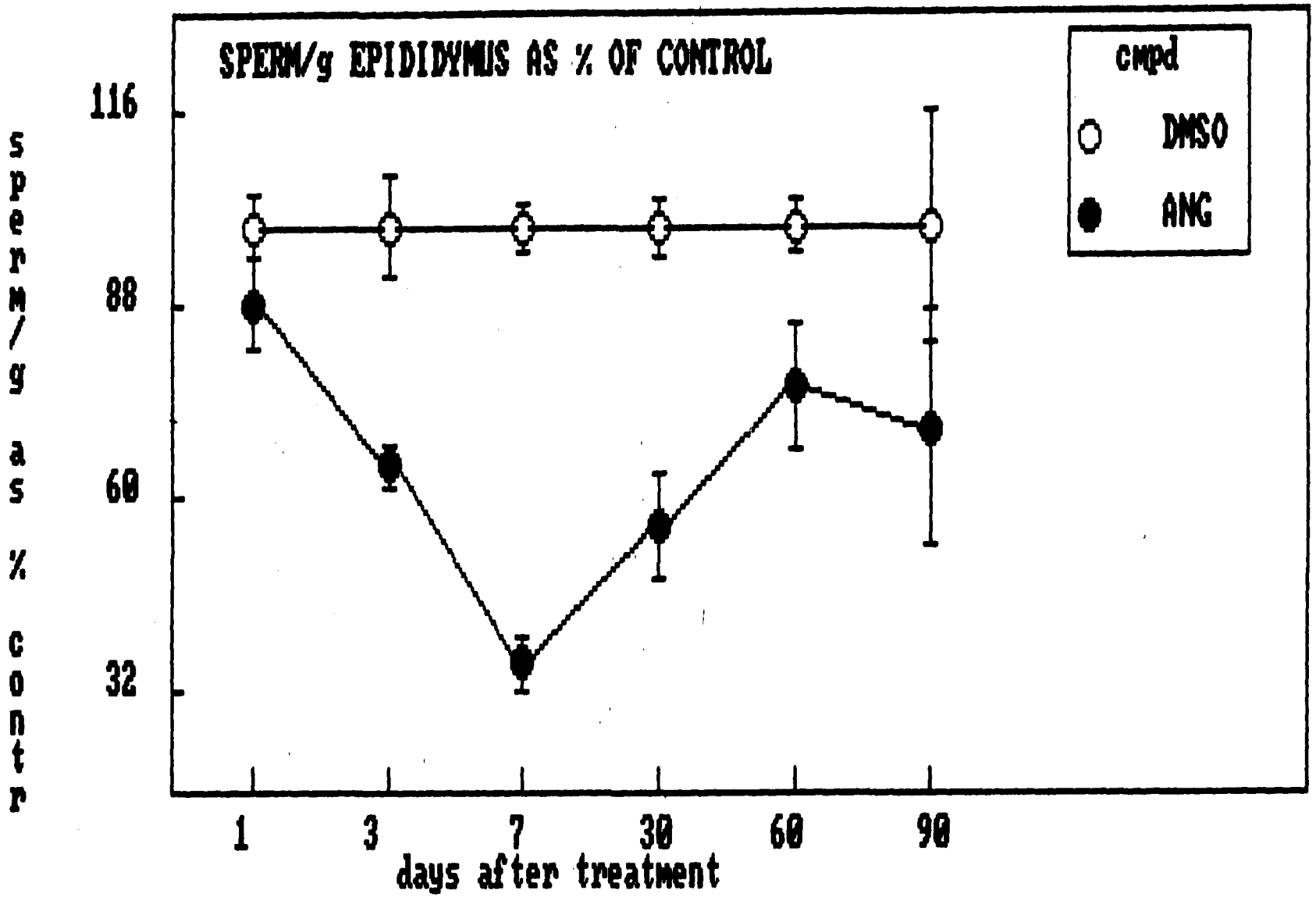
SPERM/g EPIDIDYMUS AS % OF CONTROL
cmpd
DMSO
ANG
116
88
60
32
sperm/g as % contr
1
3
7
30
60
90
days after treatment

FIGURE 11

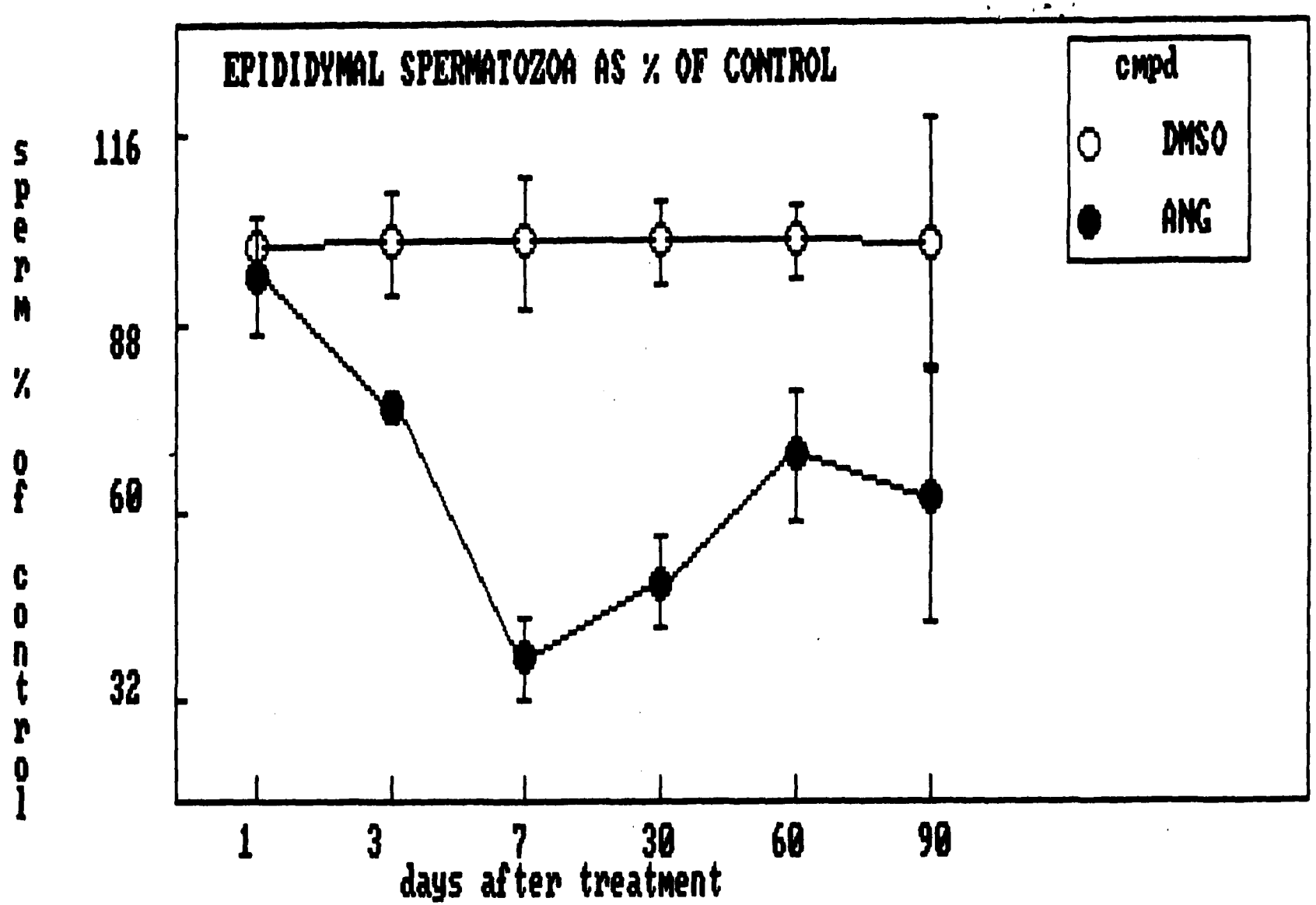
EPIDIDYMAL SPERMATOZOA AS % OF CONTROL
cmpd
DMSO
ANG
sperm % of control
116
88
60
32
1
3
7
30
60
90
days after treatment

FIGURE 12

Because regeneration of all damaged lymphoid tissues quickly followed the appearance of lesions, it is unlikely that single-dose exposure to DAS toxin would cause a lasting impairment of immune function. The transient depression perhaps in all subsets of the lymphocyte population is probably not of long-term consequence.

The observation of regeneration in the thymic cortex 4 days after treatment of mice with DAS in our studies correlates well with the reported recovery of T-cell immune function after exposure to T-2 toxin. These observations indicate that T-2 toxin and other trichothecenes are potently toxic to the immune system, but that problems in immune function are more likely to be the result of continuous exposure to trichothecene mycotoxins, rather than as a result of single dose or short-term exposure. These questions are under study in our on-going investigations.

The functional significance of the effects of DAS toxin on the mature lymphoid cells in the intestinal epithelium in the intestinal lamina propria, and in the dome regions of Peyer's patches, is unknown. These various populations of lymphocytes are generally considered to be non-germinal, differentiated lymphocytes, although they are capable of division. The reason for their susceptibility to T-2 toxin is difficult to explain; most other susceptible cells are in S phase of the cell cycle, whereas many of these intestinal lymphocytes are not. It is possible that this effect could result in impaired local intestinal immune function in animals exposed continuously to trichothecenes.

Intestinal lympholysis has not previously been recognized as an effect of DAS, probably because most of the cellular fragments disappear within hours of formation, and are difficult to visualize when fixation is less than ideal. Intestinal effects during the early stages of sublethal trichothecene toxicosis have not received detailed examination, so this effect may have been overlooked. The effects of DAS toxin on intestinal epithelial and lymphoid cells is a subject for ultrastructural examination and such studies are a part of the current contract.

The effects of DAS on the intestinal epithelium were also recognizable within a few hours after exposure to the toxin. The earliest change was the disappearance of mitotic figures, indicating that DAS prevents germinal cells from entering mitosis by arresting the cell cycle. Karyorrhexis and accompanying phagocytosis of debris by neighboring healthy cells, occurred in germinal zones of intestinal crypts within 3 hours. The severity of this early injury to intestinal crypts by DAS was dose-related, but varied among different locations in the intestine, and among different mice. The pathogenesis of changes in injured crypts was also variable. In mildly affected mice, mitotic activity resumed within 12 hours, debris rapidly disappeared within 18-24 hours, and the villous and crypt architecture was unaltered. In

more severely damaged crypts, a central cast of cell debris formed in the lumen of the crypt, and this persisted beyond 24 hours. In these mice, resumption of mitotic activity was delayed, but when crypts did regenerate, they did so in an exaggerated and disorganized manner such that villi were atrophic at 48 and 96 hours after treatment.

The transient leukocytosis observed within several hours of treatment with DAS was similar to the observation of Sato et al (1978) working with T-2 toxin, who found elevations as soon as 1 hour and peaks at 6 hours after administration of fusarenon-X, neosolaniol, or T-2 toxin to mice. These investigators speculated that this elevation was due to inflammatory reactions induced by the irritant trichothecenes. In the present study, differential counts of leukocytes 4 hours after treatment revealed neutrophilia, correlating with appearance of neutrophils in the lamina propria of the damaged intestine. Thus, the neutrophilia could be part of an inflammatory response. However, lymphocyte counts were greatly elevated within 1 hour of treatment, and remained elevated for 6 hours, with a peak at 2-3 hours. Lymphocytosis at this stage could not be a result of the stress of treatment, as suggested by Sato et al (1978), because cortisone causes lymphopenia within 4 hours. The observed lymphocytosis may have been due to the cytotoxic effects of DAS on lymphoid tissues. However, other agents such as the alkylating agents, which are similarly toxic to lymphoid tissues, cause lymphopenia within a few hours of treatment. At present, the mechanism and functional significance of the lymphocytosis induced by DAS is unknown.

Hematopoietic populations in the spleen and bone marrow also exhibited necrosis within a few hours following treatment with DAS. Numbers of circulating reticulocytes and granulocytes at 48 hours were below control values. At this stage, cells in depleted bone marrow and splenic red pulp had begun to proliferate, and colonies of immature precursors of myeloid, megakaryocytic, and erythroid cells were visible. Differentiation of megakaryocytes and segmented granulocytes was evident by 96 hours, but, at this stage, regenerating erythroid cells were still immature. These regenerating hematopoietic foci in the spleen resembled those described in mice after single exposure to radiation or to cytotoxic drugs. At 96 hours, extramedullary hematopoietic foci were evident in the hepatic sinusoids, further demonstrating the activity of the recovery phase. These observations indicate that exposure to a single dose of DAS appears unlikely to cause a lasting depression of hematopoiesis.

The CFU-S studies suggested that this might be a fruitful area for further research. It may not be of great consequence that a single exposure to DAS causes a significant, albeit transient, depression of lymphocytes to respond to stimuli in a normal fashion. However, chronic exposure, in a broader view of the problem, may be a different concern. These questions may be of

significance for future investigations, in particular, with respect to the differences in the in vivo effects which differ from in vitro cell cultures of lymphocytes exposed to DAS. It appears that the toxicity of DAS is in part a response mediated by in vivo systems probably unrelated to metabolism of the toxin.

The search for factors which may offer protection against DAS and other trichothecenes has been less fruitful than expected however some of the naturally-occurring substances may turn out to be effective when the proper mode of application is identified. It is important to find compounds which not only offer protection but materials which can be used therapeutically, after exposure has occurred.

Progress in understanding the metabolism and chemistry of DAS has been excellent. Both in vitro and in vivo routes of metabolism has been traced. It is clear that DAS is rapidly metabolized, that it is not a substrate for P-450 enzymes, and microsomal incubation with standards produced by Professor Roush's group has identified major metabolites as 4-acetoxy-scirpendiol, scirpenetriol, and 15-mono-acetoxyscirpenol. DAS is a good substrate for microsomal carboxylesterases. In vitro studies have suggested that neither epoxide hydrolase nor GSH transferase are active with DAS supporting a conclusion that the internal epoxide of DAS is not metabolized. A major conjugation is with glucuronic acid at the 3-position and with a mixture of acetoxy species at the 4 and 15 positions. These do not appear to be toxic.

In vivo metabolites have similar peaks as those identified in vitro but are not the same. While not yet determined, it appears likely that they are C4-monoacetate, C-3 glucuronide. Total analysis is in progress under the new contract. The major urinary metabolite in mice is a glucuronide with DAS rapidly hydrolyzed to a mixture of C4 and C15 monoacetates and then glucuronidation occurs at the C-3 position.

An additional aim of the metabolism segment, in vivo, was to develop a means for safe exposure by topical application and to verify the skin patch integrity. This has been accomplished and the patch successfully used in further metabolic studies. From the latter studies, using labeled DAS, it is clear that there is no major reservoir of DAS in either the mouse or the rat. In both species most of the label was found in the small intestine and in the femur. There was none in the GI contents of the mouse, a reflection of much lower levels of fecal excretion in this species. In the mouse about 80% was excreted in the urine while in the rat it was about 65%. In both species, 70% of the label was found in urine and feces within 24 hours.

The relative decreased sensitivity of the mouse compared to the rat to DAS skin application would appear to be due largely to decreased permeability in mouse skin and increased sequestering

at the application site. Histopathology of application sites support this conclusion and, that once absorbed, the sites of tissue injury and distribution and severity of lesions are the same as with other routes of exposure.

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